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This is to acknowledge that the slide shown on the front cover of this journal was kindly supplied by Dr. Caroline J. Springer, CRC Laboratory, 15 Cotswold Road, Belmont, Sutton, Surrey, SM2 5NG, UK. It shows the underlying principle of antibody-directed enzyme prodrug therapy (ADEPT) which is a 2-step therapy for cancer. The first step is the administration of an antibody-enzyme conjugate. After time for clearance from blood and normal tissues the second step, in the form of a prodrug, is injected.

†This is to acknowledge with thanks the assistance and efforts of Prof. Donald T. Witiak (University Wisconsin-Madison, Madison, USA) in the preparation of this issue.

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Aims and Scope

Current Medicinal Chemistry aims to cover all the latest and outstanding developments in medicinal chemistry and rational drug design. Each bi-monthly issue will contain a series of timely in-depth reviews written by leaders in the field covering a range of current topics in medicinal chemistry. *Current Medicinal Chemistry* is an essential journal for every medicinal chemist who wishes to be kept informed and up-to-date with the latest and most important developments.

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Myocardial Protection with the ATP-Sensitive Potassium Channel Openers

Karnail S. Atwal

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Abstract: The ATP-sensitive potassium channel (K_{ATP}) openers (e.g., Cromakalim) were originally developed for the treatment of hypertension, primarily due to their potent peripheral vasodilating properties. However, the clinical studies have failed to demonstrate any advantage of these compounds over the more established antihypertensive agents. Experimental studies have shown that the K_{ATP} openers may be effective for the treatment of a variety of diseases (asthma, urinary incontinence, ischemia etc.) besides hypertension. However, their clinical utility is limited due to lack of tissue selectivity. We have shown that K_{ATP} openers have direct cardioprotective properties which do not require contribution from vasodilation. However, the first generation agents offer a narrow window of safety for the treatment of myocardial ischemia due to their potent peripheral vasodilating properties which can compromise the tissue already at risk. Our efforts to find cardioprotective K_{ATP} openers with a lower degree of vasorelaxant potency relative to the first generation agents have resulted in the identification of a new class of agents (**5**). Their structure-activity relationships for antiischemic activity and efficacy in vivo are summarized in this review. The structure-activity studies show that an electron withdrawing group at C6 of the benzopyran ring, a gem-dimethyl at C2, a trans-hydroxyl at C3, an sp3 carbon at C3/C4 and an aryl ring attached to the nitrogen of the cyanoguanidine moiety are required for antiischemic activity. The mechanism of action of these compounds appear to involve opening of the K_{ATP} as structurally distinct K_{ATP} blockers abolish the cardioprotective effects of these compounds. Unlike the first generation compounds (e.g., cromakalim), the more selective K_{ATP} openers can be administered iv to observe antiischemic efficacy. The mechanism of antiischemic activity of K_{ATP} openers does not appear to involve the sarcolemmal K_{ATP} as their antiischemic activity is independent of action potential shortening. Progress at understanding the molecular mechanism of action of K_{ATP} openers and opportunities for future research are also discussed in this article.

Introduction

Potassium channels play a fundamental role in excitation-contraction and excitation-secretion couplings by controlling resting membrane potential. Many articles covering the basic biology and physiology of potassium channels have appeared in the literature [1]. Out of all potassium channel types, the ATP-sensitive potassium channel (K_{ATP}) is the most studied channel as a therapeutic target. This channel is known to exist in a variety of tissues including the heart, skeletal muscle, brain and pancreatic β-cells [2]. Its open probability increases as the ATP concentrations fall below physiological levels. Since ATP concentrations are linked to the metabolic state of the cell, the K_{ATP} is often referred to as a metabolically gated potassium channel. In addition to ATP, this channel is blocked by magnesium ions and certain nucleotides (ADP, AMP) [3]. In some tissues, the K_{ATP} may be regulated by adenosine receptors via G-protein coupling [4] and phosphorylation by protein kinases [5]. Although the presence of K_{ATP} in many tissue types makes it attractive as a drug discovery target, tissue selectivity becomes a major issue before advancing potential K_{ATP} modulators into clinical studies.

The agents that open as well as block K_{ATP} are of potential therapeutic utility. In fact, sulfonyl urea class of compounds that block K_{ATP} in pancreatic β-cells have been therapeutically used as antidiabetic agents for many years [6]. Their mechanism of action is thought to involve block of the K_{ATP} which causes membrane depolarization resulting in calcium entry through voltage-dependent channels. The rise in calcium concentration causes insulin release. The K_{ATP} openers on the other hand are relatively newer class of agents [7]. The primary indication for these agents was originally thought to be for the treatment of hypertension due to their potent peripheral

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vasodilating properties. Although the details of how K_{ATP} openers cause smooth muscle relaxation are not completely known, these compounds are thought to cause membrane hyperpolarization which results in raising the threshold for calcium entry through voltage regulated channels. Pharmacological studies suggest that in addition to hypertension, the K_{ATP} openers also might be useful for a variety of other diseases such as asthma, urinary incontinence and myocardial ischemia. However, the lack of tissue selectivity has restricted the clinical efficacy of existing K_{ATP} openers. Many review articles describing the basic biology of this channel and the therapeutic utility of agents that modulate its activity have appeared in the literature [8]. This review is focused on the cardioprotective actions of the K_{ATP} openers with special emphasis on cardiac selective agents. To conserve space, only selective references are provided.

Cardioprotective Properties of K_{ATP} Openers

By carrying out studies in isolated rat hearts, Grover et. al have shown that the first generation K_{ATP} openers have direct cardioprotective properties that do not involve contribution from vasodilation [9]. The cardioprotection by these agents has been shown to be a class effect as several structurally different compounds (Fig. **1**) afford cardioprotection in the 1-10 μM range. The proof for the involvement of the K_{ATP} opening in their mechanism of action continues to be the reversibility of cardioprotection by co-treatment with the K_{ATP} blocker glyburide. One advantage of the K_{ATP} openers as cardioprotective agents is their relative lack of cardiodepression at the antiischemic concentrations compared to, for example, calcium channel blockers [9]. Since most of the first generation K_{ATP} openers (Fig. **1**) have potent peripheral vasodilating properties, they carry some degree of liability with their use as antiischemic agents. The hypotension caused by these agents can result in under-perfusion of the tissue already at risk. Additionally, the myocardial action potential shortening by the first generation K_{ATP} openers can exacerbate certain types of arrhythmias [10]. Thus, a combination of powerful hypotensive effects and proarrhythmic potential of the first generation K_{ATP} openers may limit their utility for the treatment of acute myocardial ischemia.

We have been interested in exploring the utility of K_{ATP} openers as myocardial protecting agents (i.e., antiischemic agents). Our goal was to find selective antiischemic K_{ATP} openers that would protect the myocardium with minimum or no vasodilation. Since the action potential shortening by K_{ATP} openers was originally thought to be part of their mechanism of cardioprotection [9], we decided to defer that issue until we had agents that were more selective for the myocardium. Considering that ischemia is an irreversible event, the first challenge we faced was the development of a suitable model for assessing cardioprotective potencies. We settled on the isolated perfused rat heart model based on our preliminary studies with the reference K_{ATP} openers [11]. We were able to use the "time to the onset of contracture" as an index of ischemia. Time to contracture is defined as the time necessary during total global ischemia to increase end diastolic pressure by 5 mmHg. The contracture develops due to the rigor bond formation, presumably caused by the depletion of ATP. We found that the K_{ATP} openers increase time to the onset of contracture

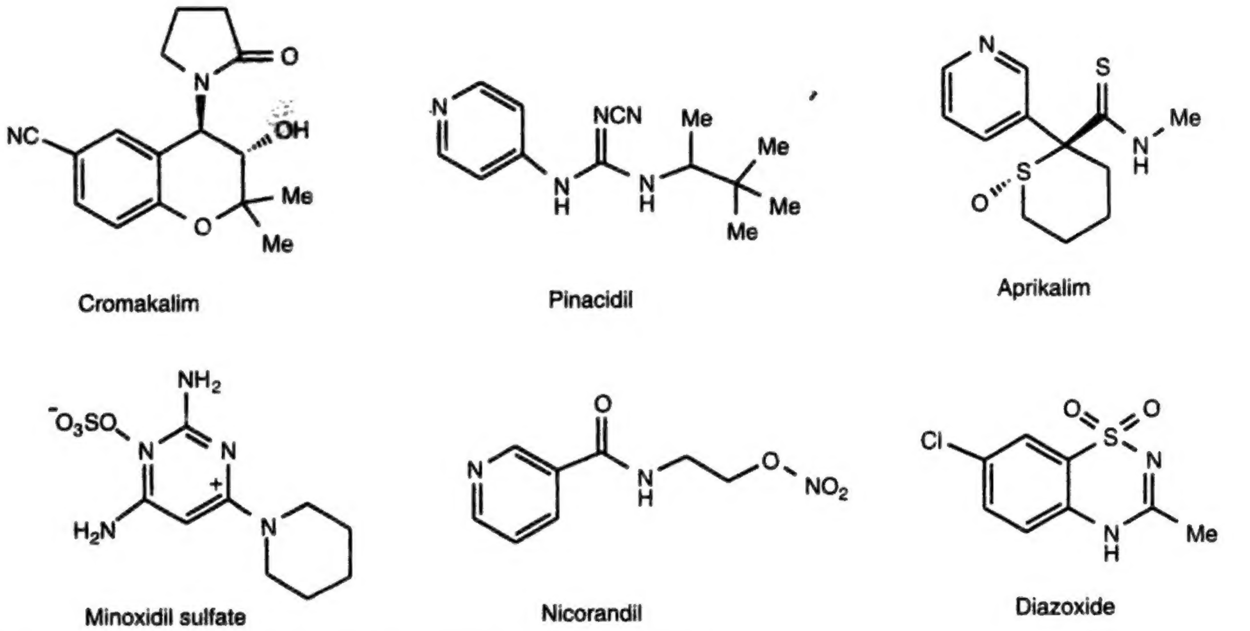

Cromakalim Pinacidil Aprikalim
Minoxidil sulfate Nicorandil Diazoxide

Fig (1). The structures of various structurally different K_{ATP} openers.

in a concentration dependent manner. The EC_{25} (concentration necessary to increase time to contracture by 25% relative to vehicle treated hearts) values determined from concentration response relationships were used to compare potencies. With some exceptions, all compounds were usually screened at 10 μM concentration. Those causing 25% or greater increase in the time to the onset of contracture were subjected to detailed concentration-response studies to determine EC_{25} values. For confirmation of mechanism, we often co-treated hearts with the K_{ATP} blocker glyburide which abolishes the cardioprotective effects of the K_{ATP} openers.

For comparison of vasorelaxant potencies, we determined IC_{50} (concentration necessary to cause relaxation by 50%) values for relaxation of the rat aortae pre-contracted with methoxamine [12]. Since the antiischemic potency is measured using isolated perfused rat hearts and the vasorelaxant potency is measured using pre-contracted rat aortae, the ratio of the two potencies does not indicate absolute selectivity. The individual tests are only useful for comparing antiischemic and vasorelaxant potencies among various test agents. The goal of our studies was to investigate if the cardioprotective and vasorelaxant activities of K_{ATP} openers had distinct structure-activity relationships. Our ultimate goal was to identify antiischemic K_{ATP} openers with lower vasorelaxant potencies compared to those of the reference agents (e.g., cromakalim).

Table 1. Vasorelaxant and Antiischemic Potencies of Cromakalim, Pinacidil and Aprikalim

Compound	Time to Contracture[a] EC_{25}, μM	Vasorelaxant potencies[b] IC_{50}, μM	Ratio EC_{25}/IC_{50}
1 (Comakalim)	8.9	0.032	278
2 (Pinacidil)	1.2	0.07	17
3 (Aprikalim)	2.3	0.04	58

[a] Antiischemic potency was determined by measurement of EC_{25}, concentration necessary for increase in time to contracture by 25% relative to vehicle treated hearts, in the globally ischemic rat hearts. Time to contracture was defined as the time necessary during total global ischemia to increase end diastolic pressure by 5 mm Hg. All compounds were intially screened at 10 μM concentration. Those causing 25% or greater increase in time to the onset of contracture were subjected to concentration-response studies to determine EC_{25} values.

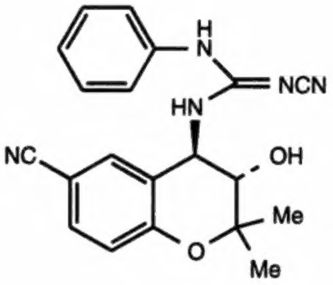
[b] Vasorelaxant potency was assessed by measurement of IC_{50} for inhibition of methoxamine contracted rat aorta.

For initial studies, we evaluated the vasorelaxant and cardioprotective potencies of reference agents cromakalim, aprikalim and pinacidil (Table 1). As expected, all compounds were potent vasorelaxant agents with potencies in the nanomolar range. Their antiischemic potencies, as determined by the EC_{25} values, were found to be between 1-10 μM range. In addition to increasing time to contracture, these compounds also enhance post ischemic recovery of contractile function and reduce lactate dehydrogenase release [13]. The cardioprotective effects of K_{ATP} openers were also demonstrated in vivo when given directly into the coronary artery to avoid complications due to hypotension [14]. Although some investigators show that cardioprotection with the K_{ATP} openers can be observed on iv administration, the window of efficacy is small [15].

Discovery of Selective K_{ATP} Openers

Our objective was to find K_{ATP} openers that maintain the cardioprotective effects of the first generation agents with lower degree of vasorelaxant activity. Although the sample size was small, comparison among the reference K_{ATP} openers indicated early on that there was no correlation between antiischemic and vasorelaxant potencies (Table 1). We carried out detailed structure-activity studies for antiischemic and vasorelaxant activities. For this work, we concentrated on benzopyran based compounds (related to cromakalim) as our preliminary studies suggested that there was a wider variation in the antiischemic potencies of this class of compounds compared to, for example, pinacidil related compounds. We found no correlation between vasorelaxant and antiischemic (increase in time to contracture) potencies. A simple conclusion to be drawn from this work was that either the structure-activity relationships for the antiischemic and vasorelaxant activities were distinct or different mechanisms were responsible for the expression of these activities. The later explanation was discounted as the vasorelaxant and antiischemic effects of these compounds were abolished by the K_{ATP} blocker glyburide. These studies and additional work led to the identification of a cyanoguanidine analog **5** which, despite being equipotent to cromakalim as an antiischemic agent, was 40-fold less potent as a vasorelaxing agent (Table 2). The difference in structure-activity relationships for the antiischemic and vasorelaxant activities was also demonstrated by evaluating the enantiomers of **5**. While the two enantiomers **6** and **7** have similar vasorelaxant potencies, most of the antiischemic potency resides in the 3S, 4R-enantiomers **6**. As demonstrated for cromakalim [9], the cardioprotective effects of **5** were completely abolished by pre-treatment with the K_{ATP} blocker glyburide (Fig. **2**), indicating that the K_{ATP} opening was involved in its mechanism of cardioprotection. These studies set the stage for

Table 2. Vasorelaxant and Antiischemic Potencies of Cromakalim, Benzopyranyl Cyanoguanidine 5 and its Enantiomers



5

Compound	Time to Contracture EC_{25}, μM	Vasorelaxant potencies IC_{50}, μM	Ratio EC_{25}/IC_{50}
5	11.0	1.4	7.9
6	6.1	1.3	4.7
7	>30[a]	4.2	-

[a] EC_{25} could not be determined due to its proischemic effects at higher than 30 μM concentrations.

further investigation of structure-activity relationships for the antiischemic activity of **5**.

Structure-Activity Relationships

The purpose of these studies was to further enhance antiischemic potency and cardiac selectivity of **5** with the goal of identifying an antiischemic K_{ATP} opener that was suitable for clinical development. Portions of this work are published [16-18]. We started with the benzopyran moiety by investigating the effect of substitution at C6 (Table 3). As shown by the cardioprotective potency **8**, removal of the C6 nitrile in **5** has detrimental effect on antiischemic and vasorelaxant potencies. The isosteric acetylene **9** is

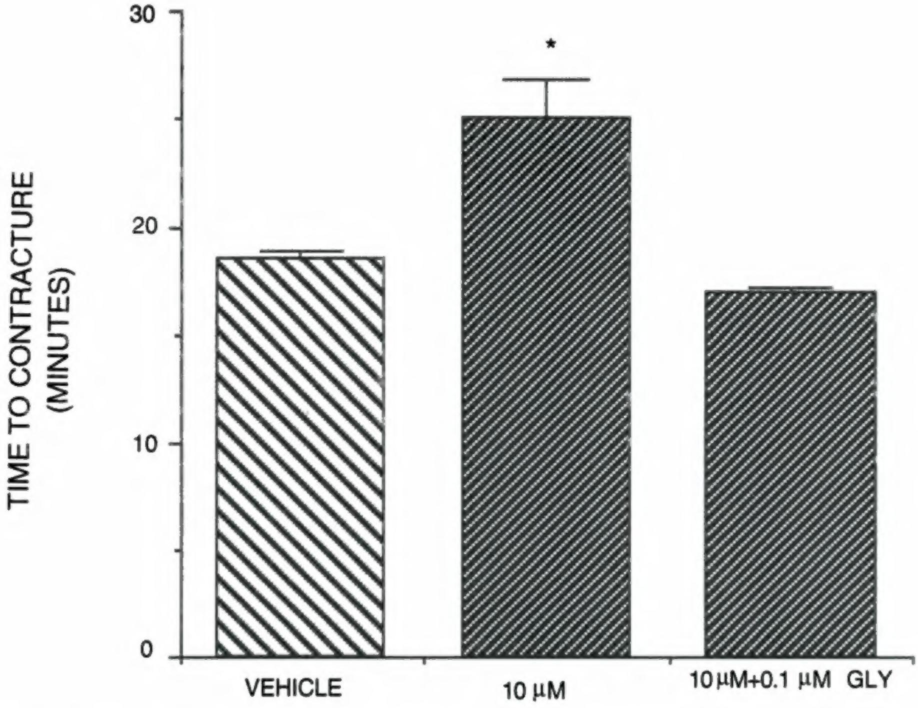
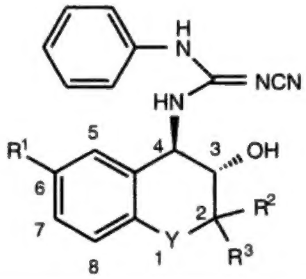


Fig. (2). Reversal of antiischemic effects (time to contracture) of phenyl-cyanoguanidine **5** (10 μM) by the K_{ATP} blocker glyburide (GLY, 0.1 μM). Gluburide abolished the cardioprotective effects of **5**.

Table 3. Vasorelaxant and Antiischemic Potencies of Benzopyranyl Cyanoguanidines; Modification of the Benzopyran Ring



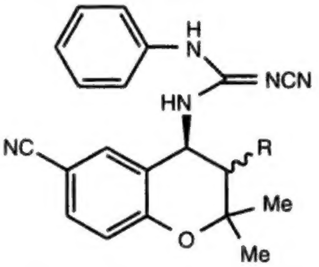
Compound	R^1	R^2	Y	Time to Contracture EC_{25}, μM or % inc. @ 10μM	Vasorelaxant Potencies IC_{50}, μM	Ratio EC_{25}/IC_{50}
5	CN	Me	O	11.0	1.4	7.9
8	H	Me	O	1%	73	-
9	CCH	Me	O	>30	5.4	-
10	NO_2	Me	O	7.2	2.0	3.6
11	CF_3	Me	O	10.0	19.2	0.5
12	$COCH_3$	Me	O	8.5	4.0	2.1
13	NH_2	Me	O	0%	59.6	-
14	NO_2	Me	single bond	10.7	0.57	18.8
15	CN	H	O	0%	194	-

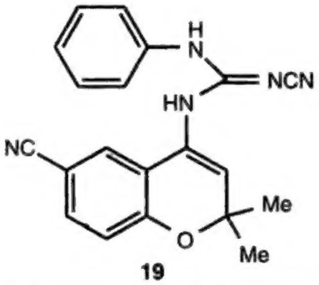
less potent than the cyano analog **5**. The presence of an electron withdrawing group was further emphasized by the antiischemic potencies of the nitro (**10**), trifluoromethyl (**11**) and acetyl (**12**) analogs. The amino analog **13** is devoid of antiischemic activity at the screening concentration of 10 μM. These data strongly support that optimum cardioprotective potency is obtained with an electron withdrawing group at C6. The oxygen atom of the benzopyran is not mandatory as its deletion (**14**) is without an effect on antiischemic and vasorelaxant potencies. The importance of the gem-dimethyl group is supported by the lack of activity in the protio analog **15** at 10 μM.

The acetyl derivative (**16**) is equipotent to the alcohol **5** indicating that the free hydroxyl group is not required for antiischemic and vasorelaxant activities (Table 4). The relative stereochemistry at C3 appears to be important as the cis analog **17** shows diminished antiischemic potency relative to **5**. Removal of the hydroxyl group at C3 (**18**) causes diminution of antiischemic potency. The lower antiischemic potency of **19** relative to **5** shows that an sp3 carbon is preferred at C3/C4.

The cyanoguanidine moiety in **5** is not mandatory for antiischemic activity as its urea (**20**) and thiourea (**21**) analogs are potent antiischemic agents (Table 5).

Table 4. Vasorelaxant and Antiischemic Potencies of Benzopyranyl Cyanoguanidines; Modification at C3 and C4 of Benzopyran





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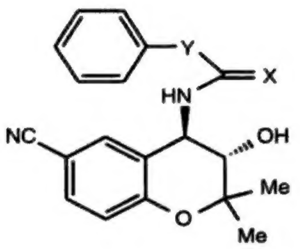
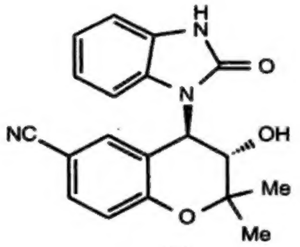
Compound	\R	Time to Contracture EC_{25}, μM or % inc. @ 10μM	Vasorelaxant Potencies IC_{50}, μM	Ratio EC_{25}/IC_{50}
5	*trans*-OH	11.0	1.4	7.9
16	*trans*-OAc	6.0	2.6	2.3
17	*cis*-OH	9%	17.0	-
18	H	0%	14.0	-
19		0%	6.9	-

Table 5 . Vasorelaxant and Antiischemic Potencies of Benzopyranyl Cyanoguanidines; Modification of the Cyanoguanidine





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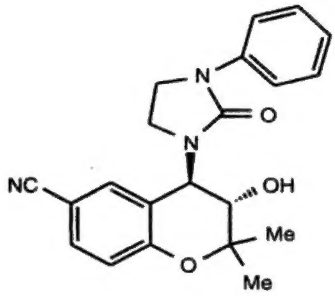

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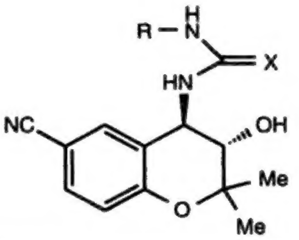
Compound	X	Y	Time to Contracture EC_{25}, μM or % Inc. @ 10μM	Vasorelaxant Potencies IC_{50}, μM	Ratio EC_{25}/IC_{50}
5	NCN	NH	11.0	1.4	7.9
20	O	NH	5.1	0.8	6.4
21	S	NH	7.7	1.7	6.4
22	NCN	O	68.8	>100	-
23	NCN	S	>30	4.9	-
24	O	CH_2	22.2	1.8	12.3
25	NCN	CH_2NH	>30	2.4	-
26	O	-	11.8	0.03	393
27			11.7	41.0	0.28
28			10.0	23.7	0.42

Replacement of the aniline nitrogen of **5** with oxygen (**22**) and sulfur (**23**) atoms has a deleterious effect on antiischemic activity. A negative impact on potency is also seen by replacement of the aniline nitrogen of **20** with a methylene group (**24**). These results suggest that a planar arrangement of the atoms connecting the pendant phenyl ring to the benzopyran ring (as in cyanoguanidine/urea) is preferred for antiischemic activity. Increasing the distance between the pendant aromatic ring and the aniline nitrogen (as in **25**) causes reduction in potency. Removal of the aniline nitrogen (**26**) has a dramatic effect on cardiac selectivity. The benzamide analog **26**, while similar in antiischemic potency to the urea analog **20**, is 30-fold more potent as a vasorelaxant. We also evaluated the effect of urea/cyanoguanidine conformation on biological potency by preparing the constrained analogs **27** and **28** of **20**. Both compounds maintain the potency of **20** with improved cardiac selectivity. These data suggest that the urea/cyanoguanidine conformation has a distinct impact on antiischemic and vasorelaxant potencies.

A negative impact on selectivity is also seen by replacement of the pendant phenyl ring with a methyl group (**29**) (Table 6). A bulky alkyl group (**30**) has a

Table 6. Vasorelaxant and Antiischemic Potencies of Benzopyranyl Cyanoguanidines; Modification of the Pendant Aryl Ring



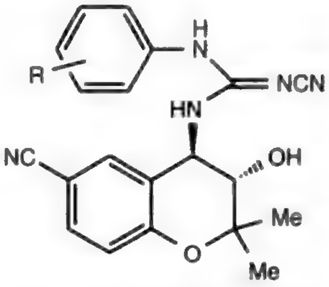
Compound	R	X	Time to Contracture EC_{25}, μM or % Inc. @ 10μM	Vasorelaxant Potencies IC_{50}, μM	Ratio EC_{25}/IC_{50}
5	Ph	NCN	11.0	1.4	7.9
29	Me	NCN	13.3	0.2	67
30	1,1-Me_2Pr	NCN	20.1	29.8	0.7
31	4-Pyridinyl	O	1%	8.5	-
32	3-Pyridinyl	O	4.2	0.5	8.4
33	2-Pyridinyl	O	2.6	0.44	5.9

deleterious effect on antiischemic and vasorelaxant potencies. These data indicate that a phenyl group might be optimum for antiischemic potency and selectivity. Replacement of the pendant phenyl ring with a pyridine is dependent on the position of the pyridine nitrogen. While the 2- (**33**) and 3-pyridinyl (**32**) analogs maintain the potency of the corresponding phenyl urea **20**, the 4-pyridinyl analog (**31**) is significantly less potent.

Since the pendant phenyl ring was found to be important for aniischemic potency and selectivity, we investigated the effect of substitution on the pendant aromatic ring (Table 7). The starting compound for these studies was the 3S,4R-enantiomer **6** of **5**. Our initial work on **6** had indicated that this compound is rapidly hydroxylated at the 4-position *in vivo*. Therefore, we concentrated on making analogs of **6** by substituting at the 3- and 4-positions of the phenyl ring to suppress hydroxylation. No trends in structure-activity relationships were seen as both the electron withdrawing (**34-39**) and electron releasing (**40**) substituents are tolerated for antiischemic activity (Table 7). The lack of cardioprotective potency in the carboxylic acid analogs **41** and **42** indicate that a negatively charged group is not compatible with biological activity. The disubstituted analog (**43**) appears to be less potent than its mono substituted counterparts **34** and **35**. The lower potency of **43** may be due to electronic rather than steric reasons as the indanyl analog **44** is equipotent to **6** as an antiischemic agent. The lower Vasorelaxant potency of **44** compared to **6** makes it one of the most selective compounds of this series.

The data summarized in Tables 1-7 show that the ratio of antiischemic and vasorelaxant potencies varies over three orders of magnitude. A correlation graph between vasorelaxant and antiischemic potencies for **45** compounds taken from our publications [16-18] in this area is shown in Fig. **3**. Lack of a correlation ($r < 0.009$) between the two potencies indicate that the structure-activity relationships for the two activities are distinct. Finding an explanation for this difference in structure-activity relationships would require further understanding of the molecular mechanism of

Table 7. Vasorelaxant and Antiischemic Potencies of Benzopyranyl Cyanoguanidines; Substitution of the Pendant Aryl Ring (all compounds chiral with 3S,4R-stereochemistry)



Compound	R	Time to Contracture EC_{25}, μM or % inc. @ 10μM	Vasorelaxant Potencies IC_{50}, μM	Ratio EC_{25}/IC_{50}
6	H	6.1	1.3	4.7
34	4-Cl	2.5	1.8	1.4
35	3-Cl	1.4	0.6	2.3
36	$3\text{-}NO_2$	5.0	0.67	7.5
37	$4\text{-}NO_2$	6.2	0.30	20.6
38	$3\text{-}CF_3$	5.3	1.6	3.3
39	$4\text{-}CF_3$	13.4	8.1	1.4
40	4-OMe	5.3	32.5	0.16
41	3-COOH	13%	>100	-
42	4-COOH	8%	>100	-
43	$3,4\text{-}Cl_2$	12.9	4.6	2.8
44	$3,4\text{-}CH_2CH_2CH_2$	8.9	41.3	0.08

antiischemic activity of K_{ATP} openers. I will return to this topic at the end of this article. Based on the structure-activity studies and additional data which is beyond the scope of this review, compound **34** (BMS-180448) was selected for further studies *in vivo*.

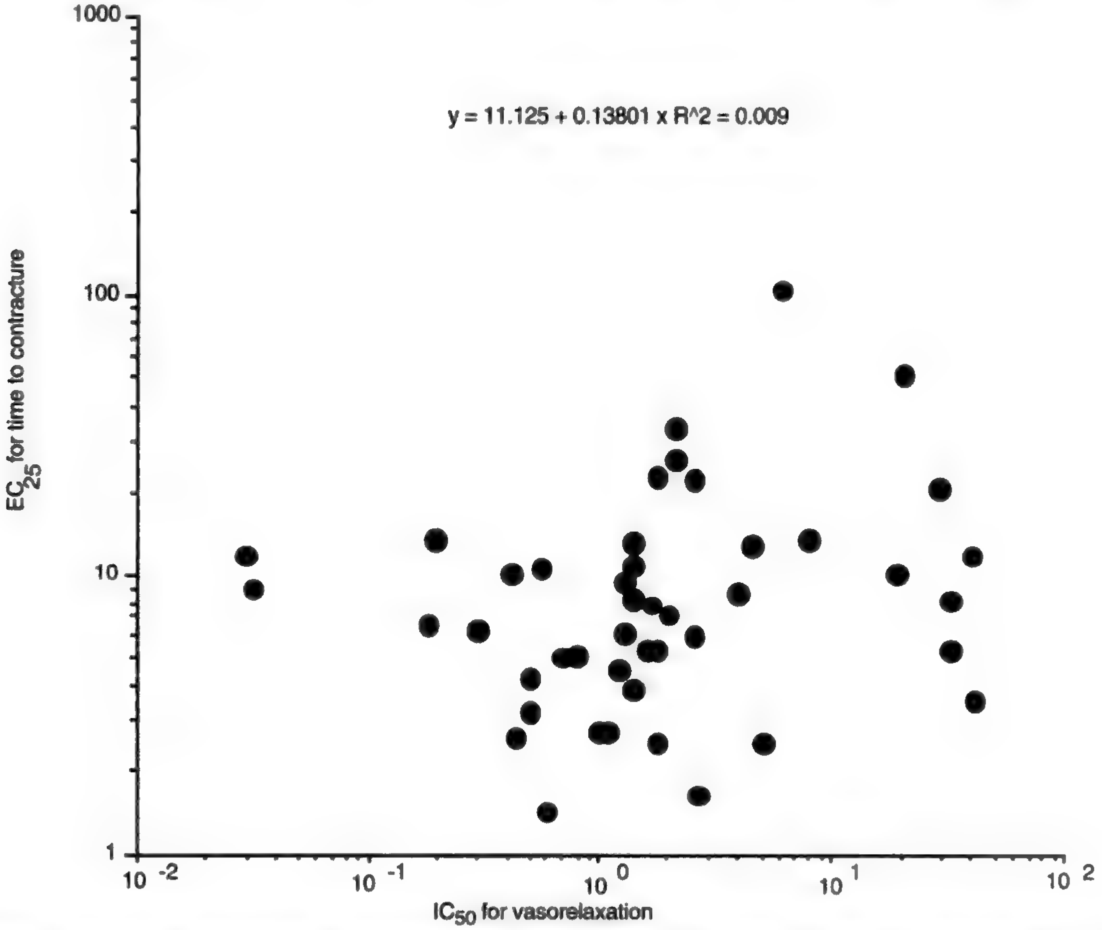


Fig. (3). Correlation between antiischemic (measured by EC_{25} values for increase in time to contracture) and vasorelaxant (measured by IC_{50} values for relaxation of methoxamine contracted rat aorta) potencies. Data for 45 compounds taken from references 16-18.

Cardioprotective Effects *In Vivo*

Earlier studies using the potent vasodilating K_{ATP} openers (e.g., cromakalim) were rather controversial. No cardioprotection was seen with pinacidil [19]. As expected, it was found to increase coronary flow resulting in coronary artery steal. Studies in canine models of ischemia carried out by Grover et. al. found cardioprotection for K_{ATP} openers (e.g., cromakalim) when these agents were directly infused into the coronary artery [14]. On intravenous administration, severe hypotension resulted before any cardioprotection could be observed. This is not surprising as the first generation agents are more potent as vasorelaxants than cardioprotectants. However, some investigators were able to show cardioprotection without hemodynamic changes [15]. Regardless, it is fair to say that the window of efficacy for the use of first generation K_{ATP} as antiischemic agents is small.

As pointed out earlier, our objective was to find more selective K_{ATP} openers that would protect the myocardium *in vivo* without potential liabilities (e.g., hypotension) of the first generation agents. The discovery of agents such as BMS-180448 made it possible to study the cardioprotective effects of K_{ATP} openers in animal models. The post ischemic recovery of contractile function and infarct size reduction were used as end-points for assessing cardioprotection in canine models of ischemia-reperfusion. While having no effect on hemodynamic variables and regional myocardial blood flow, BMS-180448 significantly reduces infarct size (expressed as the percent area at risk) in anesthetized dogs subjected to 90 minutes occlusion of the left circumflex coronary artery followed by 5 hours of reperfusion [20]. Thus, the potential complications (e.g., hypotension) associated with the use of first generation antiischemic K_{ATP} openers could be completely avoided by employing the more selective agents. The efficacy *in vivo* also has been demonstrated by the beneficial effect of BMS-180448 on the post ischemic recovery of contractile function in dogs subjected to 15 minutes of total left anterior descending coronary artery occlusion followed by reperfusion [21]. Thus, using the two in vitro test systems, we were able to identify antiischemic K_{ATP} openers that could be administered intravenously for assessment of cardioprotection in animal models of myocardial ischemia.

K_{ATP} Openers and Arrhythmias

Although K_{ATP} openers can have antiarrhythmic activity under some conditions, one potential liability of K_{ATP} openers is their ability to exacerbate arrhythmias due to their action potential shortening effects [22]. Since we felt that action potential shortening might be part of their mechanism of cardioprotection, we never fully addressed this issue at the beginning of our structure-activity studies. Further, the pharmacological tools to address this question were lacking as the first generation agents were known to shorten the duration of myocardial action potential.

Several studies published recently indicate that the action potential shortening by K_{ATP} openers and their cardioprotective effects are separable. Studies carried out with cromakalim and BMS-180448 in guinea pig hearts indicate that while the two agents have similar cardioprotective potencies, cromakalim is significantly more potent in shortening the action potential duration [23]. Thus, the two effects appear to be distinct. These *in vitro* data are also supported by studies carried out in dogs [24]. A dose of the class III antiarrhythmic agent dofetilide which has no effect on myocardial injury when given alone, was able to inhibit action potential shortening by cromakalim. The same dose of dofetilide was without an effect on the cardioprotective effects of cromakalim. These studies strongly suggest that the action potential shortening by cromakalim and other K_{ATP} openers is not required for antiischemic efficacy. Yao and Gross were also able to demonstrate cardioprotection by bimakalim in dogs without effect on monophasic action potentials [25]. Taken together, these studies indicate that the action potential shortening is not required for the K_{ATP} openers to show antiischemic activity. The simplest explanation for this lack of correlation is that the sarcolemmal K_{ATP} might not be involved in mediating the cardioprotective actions of K_{ATP} openers. The mitochondrial K_{ATP} which is involved in volume regulation might represent an alternate site of action for the antiischemic K_{ATP} openers [26].

K_{ATP} Openers and Preconditioning

The myocardial preconditioning is a phenomenon by which the heart attains the ability to protect itself from ischemic damage following short period(s) of ischemia and reperfusion [27]. The protection can be observed in many forms which include limitation of infarct size, enhanced recovery of contractile function and suppression of reperfusion-induced arrhythmias. It is a powerful endogenous protective mechanism which renders the heart more resistant to injury. The details of the mechanism of preconditioning are sketchy at this time. A large number of studies carried out in animal models [28] and man [29] suggest that the K_{ATP} opening may be the "end effector" in myocardial preconditioning, although other mechanisms cannot be ruled out at this stage. The hypothesis about the involvement of K_{ATP} opening is based on the reversibility of the beneficial effects of preconditioning with the K_{ATP} blocker glyburide [28,29]. The structurally different K_{ATP} blocker sodium 4-hydroxydecanoate is also known to abolish the cardioprotection associated with preconditioning in dogs [30]. The details of how K_{ATP} is activated following preconditioning are not completely worked out. The activation of adenosine A_1 receptors which might be linked to the opening of K_{ATP} by a second messenger system has been proposed based on the abolition by glyburide of the cardioprotective effects of adenosine A_1 agonists [31]. Regardless of how it is activated, it is clear that the opening of K_{ATP} plays a role in mediating the cardioprotective effects of preconditioning.

The cardioprotective profile of preconditioning is similar to that displayed by the K_{ATP} openers. As pointed out earlier, these agents can reduce infarct size [20], enhance post ischemic recovery of contractile function [21] and suppress reperfusion-induced arrhythmias [32]. The structurally distinct K_{ATP} blockers (glyburide, sodium 5-hydroxydecanote) completely abolish the cardioprotective benefits of K_{ATP} openers in vitro[9,20] and in vivo [33]. As demonstrated for preconditioning, the cardioprotection by K_{ATP} openers is not accompanied by cardiodepression, despite the fact that ATP levels are conserved [11]. An elegant study by Yao and Gross shows that the threshold for preconditioning is reduced by administration of a sub-threshold dose of the K_{ATP} opener bimakalim [34]. This result is expected only if bimakalim and preconditioning were exerting cardioprotection by a similar mechanism as a cardioprotectant acting by a different mechanism would reduce the efficacy of preconditioning. Thus, it is tempting to speculate that the K_{ATP} openers might be mimicking the cardioprotective effects of preconditioning. It is also important to point out that this effect of the K_{ATP} openers is not unique to the myocardium as recent studies by Lazdunski's group show that the K_{ATP} openers mimic the beneficial effects (e.g., neuronal protection) of cerebral ischemic preconditioning in hippocampal neurons [35]. As for the myocardium, the protective effects of sub-lethal insults in hippocampal neurons appear to be mediated by the activation of adenosine A_1 receptors which results in the opening of K_{ATP} [35]. Thus, it is not surprising that the K_{ATP} openers have been hypothesized to be the "chemical preconditioning agents" [36].

Prospects

Two major issues that need to be addressed are the potential clinical utility of cardioprotective K_{ATP} openers and their molecular mechanism of action. Experimental studies suggest that the cardioprotective K_{ATP} openers might be useful for indications such as acute myocardial infarction, adjunctive therapy to cardioplegia and chronic stable angina. The nature of some of these indications would require risk taking on the part of the pharmaceutical companies to pursue these compounds in the clinic. Another limitation is the exclusion of type II diabetics who are being treated with the sulfonyl urea class of antidiabetic agents. Some of these patients are also at risk for heart disease. The only agent that is currently being used for the treatment of angina pectoris is the nitro vasodilator nicorandil. It is also one of the least potent cardioprotective K_{ATP} openers. The contribution of K_{ATP} opening activity towards its overall antianginal profile is hard to estimate.

The tests currently employed to identify more potent and selective antiischemic K_{ATP} openers have a relatively low throughput which explains the non-existence of structure-activity data prior to the publication of our own work [16-18]. These tests are based on the functional activities of these compounds and consequently, they are not amenable to high volume screening. In order to set up high throughput tests to identify structurally distinct compounds with improved profile of activity (potency, selectivity), we need to understand the molecular details of their mechanism of action. An understanding of the molecular organization and regulation of the K_{ATP} in different tissues and sub-cellular compartments is bound to shed some light on the molecular basis of tissue selectivity.

The most important tools for investigating the mechanism of antiischemic activity of K_{ATP} openers continue to be the sulfonyl urea class of K_{ATP} blockers. As demonstrated by many investigators, the cardioprotective effects of K_{ATP} openers are abolished by pre-treatment with the K_{ATP} blockers glyburide and sodium 5-hydroxy decanoate (Fig. **4**) [20]. It is now clear that sulfonyl urea receptor is an integral part of the K_{ATP} channel complex [37]. However, the details of how the sulfonyl ureas and the K_{ATP} openers interact at

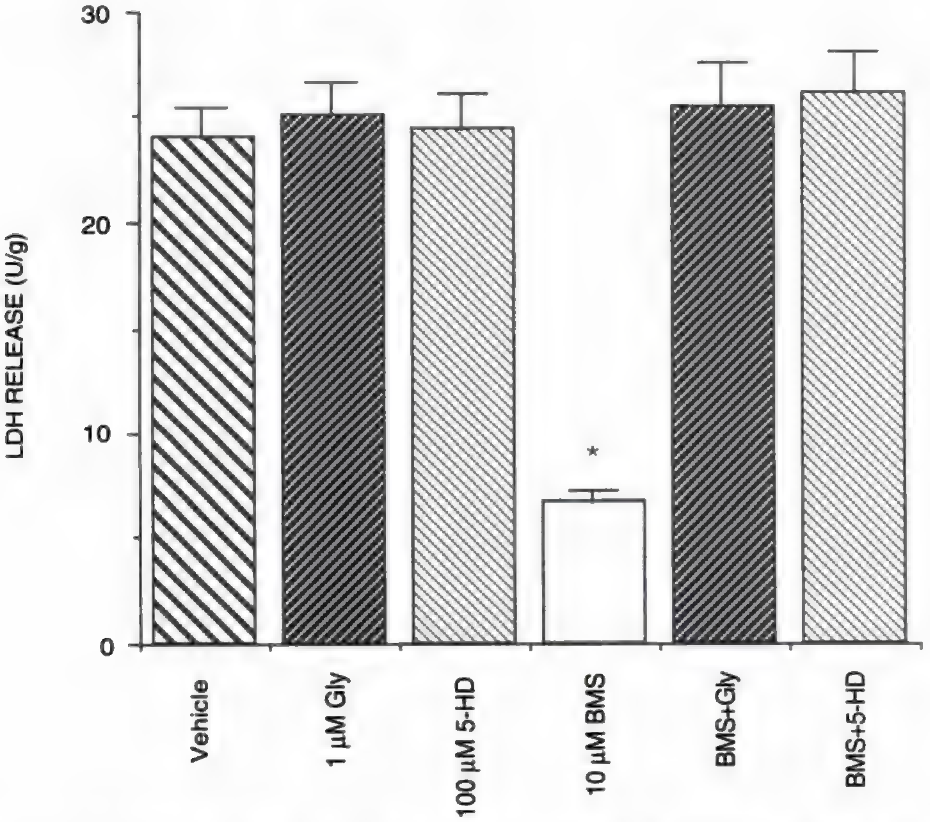


Fig. (4). The effect of K_{ATP} blockers glyburide (Gly) and sodium 5-hydroxydecanoate (HD) on the reduction of lactate dehydrogenase (LDH) release by BMS-180448 in globally ischemic rat hearts. Both glyburide and sodium 5-hydroxydecanoate abolished the cardioprotection (as shown by LDH release) afforded by BMS-180448. *Significantly different from vehicle treated hearts.

the receptor level remain to be worked out. Any system that is used to investigate that interaction has to take into account the studies indicating that the cardioprotection by K_{ATP} openers might not be mediated by opening of the sarcolemmal K_{ATP} [26].

Glyburide is known to have a high affinity binding site in several tissues [38]. Other than a potential allosteric interaction, K_{ATP} openers do not compete for the binding site occupied by glyburide. The binding site for the K_{ATP} openers has been very difficult to demonstrate in membrane fractions. A high affinity binding site for the pinacidil analog P-1075 has been demonstrated in whole cells[39] and intact tissues [40]. The binding affinities appear to correlate with the potencies of selected K_{ATP} openers to cause ^{86}Rb efflux and smooth muscle relaxation, indicating that the binding site may be relevant to the smooth muscle relaxation in some tissues [40]. However, the binding site is difficult to localize in subcellular fractions indicating that factors other than membrane proteins might be involved in regulating the binding site(s). Demonstration of a high affinity binding site(s) in a relatively high throughput system (e.g., membrane based) would be an important step forward in understanding the mechanism of action of K_{ATP} openers. The relevance of a cardioprotective site for K_{ATP} openers has to be validated by a correlation between their binding affinities and cardioprotective potencies.

The reconstituted systems that involve the cloned inward rectifier protein and the sulfonyl urea receptor could be very useful in investigating the mechanism of action of K_{ATP} openers. Recent studies by Inagaki et. al show that the pancreatic β-cell K_{ATP} contains at least two subunits - a protein BIR (kir6.2) of the inward rectifier family and the sulfonyl urea receptor (SUR), a member of the ATP-binding cassette protein [37]. Coexpression of the kir6.2 and SUR generates potassium currents with characteristics similar to those of the native K_{ATP} in β-cells. When expressed alone, kir6.2 cannot conduct ions. The SUR confers ion channel activity and nucleotide sensitivity to the channel. The co-expressed channel is blocked by sulfonyl ureas and opened by the K_{ATP} opener diazoxide. The details of the exact number of subunits of SUR and kir6.2 in the channel complex and their molecular association is not completely worked out at the present time. It is also not known if the binding of K_{ATP} openers require both kir6.2 and SUR proteins. Nevertheless, the studies by Inagaki et. al. represent a major breakthrough in our understanding of the molecular details of K_{ATP} and its modulation by small molecules. A cartoon depicting the possible interaction of K_{ATP} openers with the channel is shown in Fig. **5**. A cell based system coexpressing the cardiac inward rectifier protein and the SUR could provide an important vehicle to study the interaction between the sulfonyl urea class of compounds and the K_{ATP} openers. An important step in that direction would be

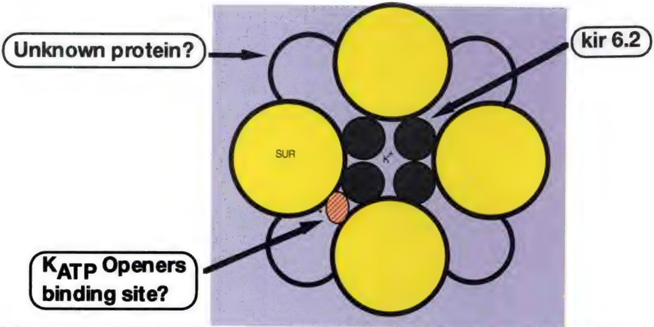


Fig. (5). A cartoon depicting the potential molecular architecture of K_{ATP}. The channel is composed of the sulfonyl urea receptor (SUR), an inward rectifier protein (kir 6.2) and some (if there is any) unknown protein(s). The details of where K_{ATP} openers bind are unknown at the present time. The cartoon is based on the findings of Inagaki et al. in the insulin secreting β-cells (refernce 37) and is not meant to represent the actual number of SUR and kir 6.2 subunits present in the channel.

the demonstration of a high affinity binding site for the K_{ATP} openers in the reconstituted system.

In summary, it is clear that work over the last few years has provided major insights into the molecular details of the K_{ATP} and its potential regulation [37]. It is only a matter of time before we understand the details of the mechanism of action of K_{ATP} modulators. It took over 20 years to figure out the identity of the SUR and its relationship with the overall molecular architecture of the K_{ATP}. This was all made possible by a combination of the powerful techniques of molecular biology and electrophysiology. I am very optimistic that in the near future, we will know more about the molecular details of the K_{ATP} in different tissues and sub-cellular compartments which could lead to a better understanding of tissue selectivity at the molecular level.

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I wish to express my sincere gratitude to my colleagues (names in references) for their contributions to the field of ion channel research, in particular the K_{ATP} and its role in myocardial protection. Without their support and collaboration, I would not have been able to contribute this article.

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Pyrrolic Photosensitizers

E. Sternberg and D. Dolphin*

This review is dedicated to the fond memory of Rosalia Frydman, Scientist, Colleague and Good Friend

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Abstract: There are three major classes of photosensitizers which can initiate a phototoxic effect to tissues upon irradiation. These are the cationic dyes (Type I Photosensitizer), the psoralens (Type I and II Photosensitizer), and cyclic polypyrrolic compounds (Type II Photosensitizer). The polypyrrolic compounds such as porphyrins have been investigated since the late 1800's for their ability to destroy tissue. In the last ten years, compounds such as chlorins and expanded porphyrins have begun to dominate the preclinical field because they can be activated with light at wavelengths tissue does not effectively absorb. The results of these investigation have been reported in more than 10,000 papers in the field covering aspects from mechanisms of action, photophysics, structure activity relationships, new compound development and clinical investigations. With the approval of PHOTOFRIN®, (porfimer sodium), the first of the pyrrolic compounds to win acceptance by international regulatory bodies and with nearly 10 other second generation compounds undergoing early clinical trials for diseases ranging from esophageal and skin cancers to age related macular degeneration, the field of photodynamic therapy would seem to have a bright future.

Introduction

Photodynamic therapy (PDT) proceeds by the introduction of a photosensitizing drug into a patient followed by irradiation with visible light to initiate treatment of diseased tissue [1]. This review will address the history of PDT along with the basic issues involved in the development of photosensitizing pyrrolic macrocyclic based drugs. This is an especially auspicious time for our review because of the recent approval of the flagship drug of QLT PhotoTherapeutics, PHOTOFRIN®, (porfimer sodium) as the first PDT agent, by the FDA for late stage esophageal cancer. The previous approval of this drug in Japan for the treatment of early stage esophageal, lung, stomach, cervical cancers as well as cervical dysplasia, in Canada for bladder cancers and esophageal cancers, in Holland and France for esophageal and lung cancer from other boards of health in Europe have established PDT as a fourth modality in the treatment of solid tumors [2].

The utilization of a drug and light in combination goes back to the ancient Egyptians who used a plant derived psoralen and sunlight to treat vitiligo. At the turn of the century it was recognized by Raab, while working in Tappeiner's laboratory, that paramecia could be killed by light in combination with an acridine dye [3]. Later, Hausman showed that mice were sensitized to light after injection of a porphyrin sensitizer [4]. The first experience of a human with such a phenomena occurred in 1913, when Meyer-Betz photosensitized himself for several weeks with a preparation of hematoporphyrin dihydrochloride [5]. This sensitization reflected the detrimental effects seen with the some of porphyrias. Hematoporphyrin (Hp, Fig. (**1**)) prepared by the method of Nencki and Zaleski [6] was shown by Schwartz [7], during the 1950's, to vary extensively from batch to batch in its ability to accumulate and exhibit fluorescence in tumors; a phenomenon first observed by Frigge [8]. This variability initiated the development of a consistently active compound by treating hematoporphyrin dihydrochloride with first sulfuric acid in acetic acid followed by base saponification, Fig. (**1**). This hematoporphyrin derivative (HpD) was found to be more consistent in its accumulation behavior in tumors.

This led Lipson in his masters thesis, during the early 1960's under the direction of Schwartz, to investigate more fully the phenomena of sensitization and fluorescence [9]. Experiments of note were the

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observation that intraperitoneal injection, as compared to intravenous, followed by light irradiation showed no major sensitization for the first process while the later caused death of mice. Another interesting observation was that prednisalone injected the day before therapy caused an increase in survival upon intravenous injection of HpD which was followed 1 hour later by irradiation [9]. He also noted that there seemed not to be a similar protective response to photosensitivity by histamine. Lipson's major concern during his experimental protocols was trying to find methods to limit the effect of sensitization in skin so that fluorescence would be a useful technique for the observation of tumors. At the time, the amount of drug injected per gram of mouse exceeded 0.15 mg. This large amount was required to observe reasonable fluorescence with the technology of the day.

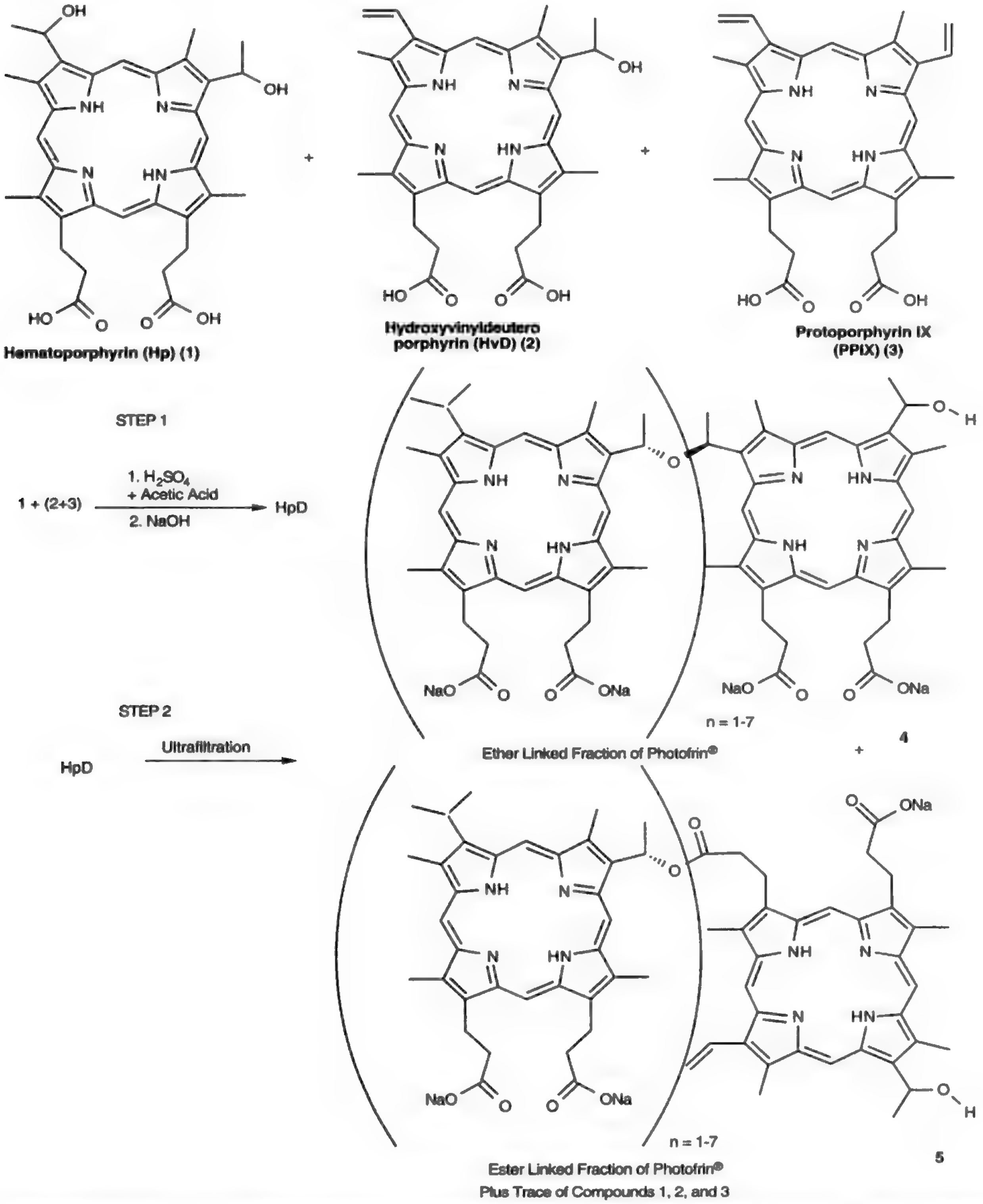

Hematoporphyrin (Hp) (1)
Hydroxyvinyldeutero porphyrin (HvD) (2)
Protoporphyrin IX (PPIX) (3)
STEP 1
1 + (2+3)
1. H2SO4 + Acetic Acid
2. NaOH
HpD
STEP 2
HpD
Ultrafiltration
n = 1-7
Ether Linked Fraction of Photofrin®
4
+
n = 1-7
Ester Linked Fraction of Photofrin®
Plus Trace of Compounds 1, 2, and 3
5

Fig. (1). Hematoporphyrin (Hp), Hydroxyethylvinyldeuteropoprhyrin (HvD) and Protoporphyrin IX (PPIX) are all components of crude hematorporphyrin dihydrochloride. Treatment with sulfuric acid in acetic acid followed by base makes either ether or ester linkages.

Lipson, during his observation of fluorescence *in-vivo* in tumors, was perhaps the first to systematically utilize a filtered irradiation system. His investigation, in rats and mice, with two tumor cell lines showed that after injection of HpD, fluorescence increased and stabilized in the tumor at ~2 hours. The tumor remained distinct from that of surrounding tissue for up to 24 hours after injection. His conclusions were that a diagnosis of neoplasia might be possible, and that delineation of the tumor at the time of surgery was viable.

Many areas of interest for today's researchers were initially investigated by Lipson and his colleagues. In fact, these still currently form the majority of research areas in the field. He was one of the first to confront the problems of incident light and its spectrum, the absorption spectrum of the drug in relationship to incident radiation intensity, the concentration of the drug in the tumor at various times after injection, the level of injected drug versus its toxicity, the amount of light required to cause necrosis of the skin, the immunological responses to the exhaustive irradiation which resulted in edema, the photophysics of fluorescence versus absorption, the photophysics of sensitization of oxygen in a biological environment, and finally the best ways to observe the fluorescent emission [10].

Lipson was also aware of the relative inability of 'pure' hematoporphyrin to accumulate in tumors and to exhibit a severe photodynamic response at the same concentrations as HpD. He was also aware of the earlier observations of Vannotti [11] that the more hydrophilic porphyrins such as uroporphyrin (octacarboxylic acid) and coproporphyrin (tetracarboxylic acid) [12] are more phototoxic to skin than hematoporphyrin, or protoporphyrin IX, Fig. (**1**) thus displaying a rudimentary knowledge of structure versus activity. There was one glaring omission in Lipson's thesis . He never mentions that treatment of a cancer would be possible upon irradiation only at the site of the tumor. A few years hence he was the first to address this concept by treating one patient with metastatic breast cancer [13]. However, this was only reported as an abstract from a conference.

Modern day PDT began with the first systematic treatment of a patient by Simons et al. [14] in the early 1970's followed shortly thereafter by Dougherty [15]. Both groups used HpD. Lipson et al continued in the field concentrating on the fluorescent detection of metaplasia and dysplasia and carcinoma *in situ* utilizing an HpD made by Wilson Pharmaceutics of Chicago [16] Lipson tried to pursue this methodology further but was hindered at the time by technological limitations of light sources and methods of detection. The first report of an 'official' Phase I/II trial utilizing an in house produced HpD and filtered light from xenon-arc lamp was reported by Dougherty's group in 1979 [17]. As we understand it, this material began to be supplied to other researchers in the field during the late 70's and early 80's and was eventually designated, PHOTOFRIN I. Dougherty et al also investigated at this time the drug for the treatment of animals [18].

The structure of the active components of HpD is still in essence under debate to this date. It was recognized early, by thin layer chromatography and then in the early 1980's using HPLC, that the mixture contained ~50% of the starting material porphyrins - hematoporphyrin, hydroxyethylvinyldeuteroporphyrins and protoporphyrin IX [19] along with other species. Injection of these relatively pure monomeric porphyrins into animals did not show selective uptake into neoplastic tissue and they were found to be rapidly excreted. The first reasonable and largely correct description of this material was given by Bonnett and Berenbaum [20] in 1981 in which they described the material as an oligomeric mixture of ether (**4**) and ester (**5**) linkages, Fig. (**1**), with the possibility of C-linkages through the methine position of the porphyrins. They justified their analysis based on purely chemical grounds in that the acetic acid/sulfuric acid treatment would result in a diacetate of the hematoporphyrin alchohols. Activation of this species under basic conditions should result in formation of ethers and esters. It was further suggested that the dimeric esters would be more difficult to hydrolyze than the original acetates. They isolated, by HPLC chromatography, a highly retained fraction which seemed to accumulate to a greater extent in their animal model. They then made a statement with which any medicinal chemist has to agree. "Finally, it seems appropriate to suggest that in future biological and clinical trials, it would be preferable to use a single well defined substance...." This did not immediately occur. Shortly thereafter; Dougherty and his colleagues described a method utilizing ultrafiltration to purify the active component to ~90% purity on a relatively large scale from HpD [21]. The patent describes this material as being largely a dimeric ether derivative based on the fact that mass spectral analysis failed to see higher oligomers in any quantity. This material was designated PHOTOFRIN II. Soon thereafter Johnson and Johnson in collaboration with the Oncology Foundation of Buffalo began official clinical trials with PHOTOFRIN II. During the mid-1980's, Dougherty was the most important champion in promoting the advancement of PDT as a therapeutic modality and is clearly the "Father of Photodynamic Therapy". At some point before 1987 Johnson and Johnson felt that pursuing this drug, the only oncological one in their pipeline, was not in its future corporate plans, so it agreed to sell the rights of PHOTOFRIN II to Quadra Logic Technologies (now QLT PhotoTherapeutics).

The structural investigations into PHOTOFRIN II, now called PHOTOFRIN®, at the time of invention included mass spectroscopy of the methylated drug and carbon-13 analysis of the material in deuterated DMSO. Modifications were introduced by American Cyanamid and QLT on the original material which accounts for a further patent issued in 1989 [22]. These consisted of longer base treatment to decrease residual ester linkages along with a lyophillization procedure for the purified product derived from ultrafiltration. Both of these processes greatly improve consistency between batches compared to the original PHOTOFRIN II.

Regulatory approval first came in Canada in 1993 and was soon followed by approval for sale in Japan in 1994. Alos QLT took back its rights to PHOTOFRIN® in North America, and the worldwide rights to BPD, Fig. **(7)**, and pursued the U.S. NOA submission for the treatment of late stage esophageal cancer on its own. Because of the nature of this complex mixture, the submission required an additional mass spectral assay of the type reported by Pandey et al. [23] which showed oligomers out to at least octamers and a capillary zone electrophoresis assay which was inspired by a paper by Sweedler and Wu [24]. The FDA approval of this submission in 1995 was followed by a new corporate agreement between QLT PhotoTherapeutics with Sanofi Winthrop Inc., for the rights to sell PHOTOFRIN® in the U.S.

As Bonnett mentioned, photosensitizers utilized for clinical trials should ideally be pure. There are other properties which have been learned about such drugs since the time of his statement. The first of these is a better spectral footprint than that of PHOTOFRIN®. The second is to optimize distribution into the region of tumor by utilizing information derived from standard oncological drug studies. Lastly, the drug should have a limited lifetime in the skin, an organ with unusualy high affinity for any drug, to minimize the period of shin photosensitivity.

Photophysics, Irradiation and Mechanisms

Compounds with extended aromatic systems such as porphyrins (P) absorb light with high efficiency, Figs. **(2)**+**(3)**. The process of dissipating the energy to its environment allows for several photophysical processes. As shown in Fig. **(2)** a photo-excited state species formed by the absorption of a photon generates an excited sensitizer. This diradical species can interact with other compounds via an electron transfer mechanism (Type I -photoprocess) to destroy or add to them and photobleach themselves, or intersystem cross from the singlet to the triplet state. This triplet can interact with ground state triplet oxygen to give singlet oxygen and the ground state of the photosensitizer. The products derived from reaction with of singlet oxygen are defined as Type II photoprocess. The triplet of the sensitizer can also again behave like a diradical to undergo electron transfer reactions or add directly to a double bond system, as is seen with psoralens [25].

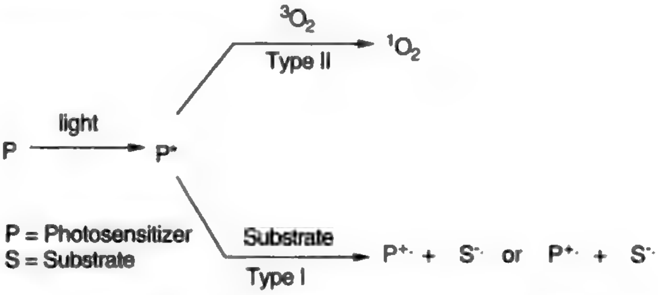
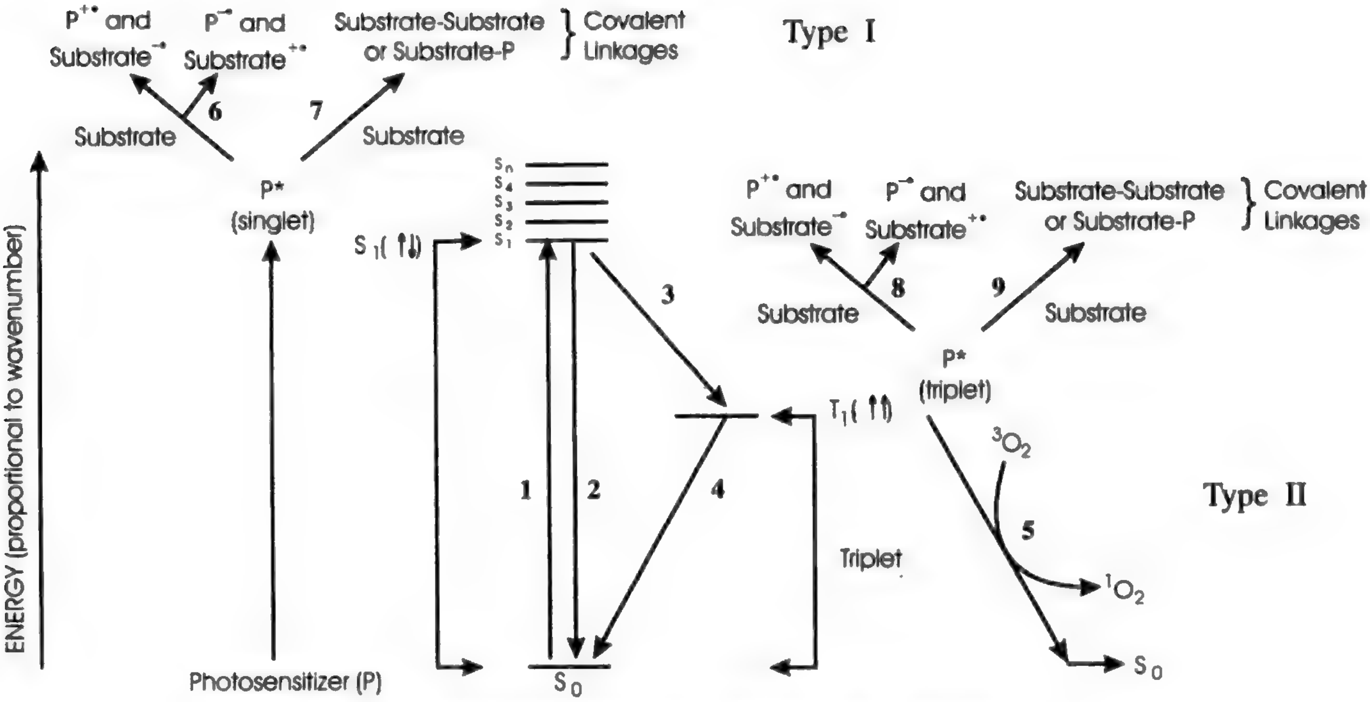


Fig. (2). Type I photoreactions are the result of a photoexcited state of a sensitizer interacting via either electron transfer or crosslinking with a substrate. Type II photosensitizers transfer energy to triplet oxygen to give singlet oxygen which then reacts with substrate.

Knowledge of photophysical properties of a new singlet oxygen photosensitizer such as quantum yields for fluorescence, singlet oxygen production and photobleaching is important during its initial evaluation [26]. It is known that certain photophysical properties make for an ideal photosensitizer of singlet oxygen. First, light must be available to it in the body. Unlike the psoralens which absorb light in the near UVA region of the spectrum, compounds which are successful as PDT agents absorb light in the near red region. This region is ideal in that light penetrates tissue most efficiently at these wavelengths [27]. It is advantageous that the compound should have a high extinction coefficient of absorption. This allows a greater percentage of the light to be absorbed. The sensitizer should sensitize singlet oxygen production with a high quantum yield. This makes for a catalytic cycle in which this reactive form of oxygen accounts for a majority of the toxicity [28]. Finally, it has been noted that a photosensitizer which photobleaches at a low but reasonable rate in tissue provides a better overall PDT effect [29].

It is best to understand these processes in light of a Jablonski Diagram, Fig. **(3)**. The photophysical data for the processes represented in this diagram can be generated in a day for a single compound. Redmond and his colleagues [30] in their studies on derivatives of the second generation drug BPDMA, Fig. **(4)**, have shown that the molar extinction coefficient, which is a measurement of the ease of a compound to reach the first singlet excited state for the 688 nm peak, ranges from 44,000 in benzene to 34,000 in methanol. The lifetime of this excited state species in methanol is 5.2 ns while its quantum yield for fluorescence is ~0.03


$P^{+\bullet}$ and Substrate$^{-\bullet}$
$P^{-\bullet}$ and Substrate$^{+\bullet}$
Substrate-Substrate or Substrate-P
Covalent Linkages
Type I
6
7
Substrate
Substrate
P* (singlet)
S_n
S_4
S_3
S_2
S_1
S_1(↑↓)
ENERGY (proportional to wavenumber)
1
2
3
4
Photosensitizer (P)
S_0
$P^{+\bullet}$ and Substrate$^{-\bullet}$
$P^{-\bullet}$ and Substrate$^{+\bullet}$
Substrate-Substrate or Substrate-P
Covalent Linkages
8
9
Substrate
Substrate
P* (triplet)
T_1(↑↑)
$^3O_2$
5
$^1O_2$
S_0
Triplet
Type II

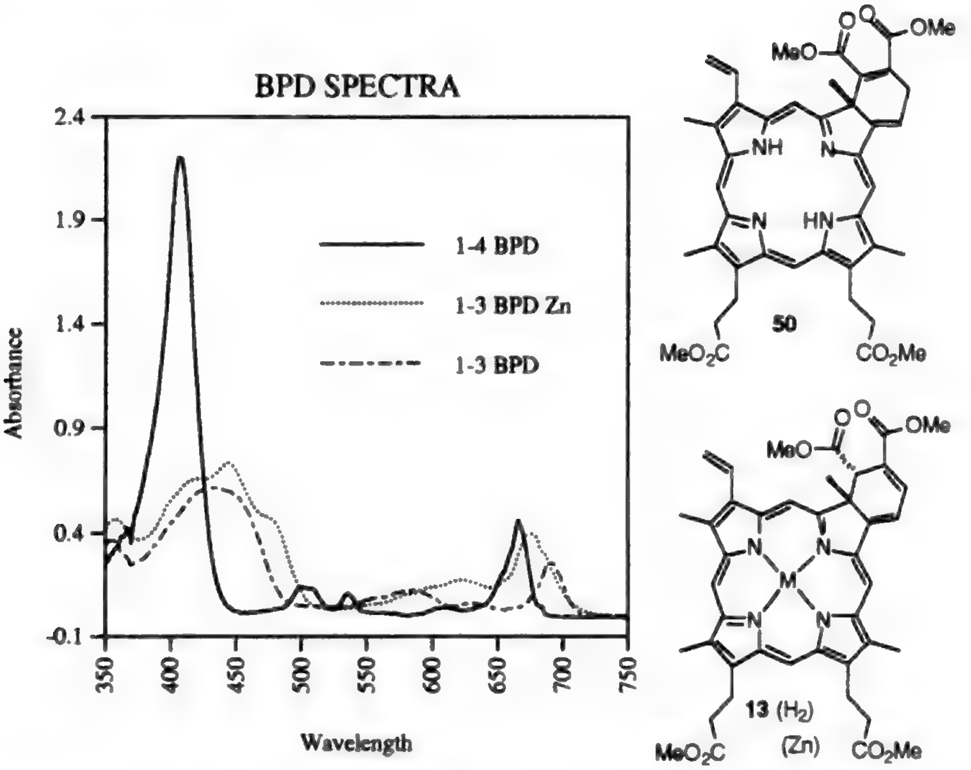
1. Absorption - Depends upon extinction coefficient and wavelength
2. Fluorescence - Lifetime depends on molecular interactions
3. Intersystem crossing
4. Phosphorescence
5. Conversion of triplet oxygen ($^3O_2$) to singlet oxygen ($^1O_2$)
6. Electron transfer from singlet state to give substrate and photosensitizer free radicals
7. Covalent bond formation between substrates or substrate / photosensitizer
8. Electron transfer from triplet state to give substrate and photosensitizer free radicals
9. Covalent bond formation between substrates or substrate / photosensitizer

Fig.(3). Jablonski Diagram.

(3%). Intersystem crossing efficiency is 0.79 to give a triplet whose lifetime exceeds 25 microseconds. The singlet oxygen quantum efficiency ranges to 0.76 while the photobleaching yield in an organic solvent is less than 6.0 x 10^{-5}. These later two numbers are important in the evaluation of a new drug in that they indicate its effectiveness as a photosensitizer and its stability. These results indicate that for every 10^5 singlet oxygen molecules produced approximately 1 photosensitizer molecule is destroyed.

The transfer of these points of information to a biological setting is difficult in that it is known that the lifetime of singlet oxygen, resulting from irradiation of a sensitizer in an aqueous biological system, is sufficiently short to the point that it cannot be readily measured [31]. As an example, in the study by Redmond, the evaluation of the photobleaching of this drug in an aqueous solution containing 10% fetal calf serum showed a nearly three fold increase in photobleaching to 2 x 10^{-4} [30]. Nearly all the compounds which presently are undergoing clinical trials have good quantum yields for the production of singlet oxygen in organic solvents [32-34]. However, such processes are difficult to evaluate in a biological system because of the effect of regional quenching and bleaching by biological species. More direct evidence on the phototoxicity to a cell line along with addition of singlet oxygen scavengers such as dimethylfuran [35] seem to be useful for an evaluation of the final effectiveness of a phototoxin once it distributes into cells.

The result of the production of singlet oxygen in a biological system can only be partially evaluated. It results in processes which resemble oxidative stress. As compared to this, there is a great deal known about the photoproducts resulting from the addition of singlet oxygen to biological compounds such a nucleic acids [36], amino acids and peptides [37] and lipids [38], Fig.



50

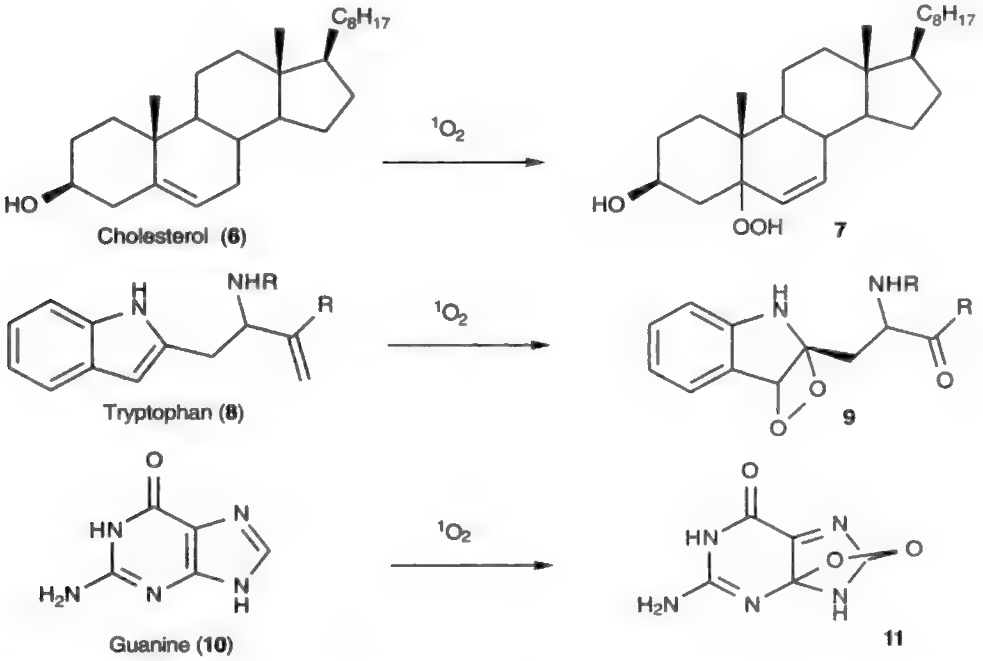
13 (H₂) (Zn)

Fig. (4). Shown is the visible spectra of benzoporphyrin derivative-1,4-diene, benzoporphyrin derivative-1,3-diene and its Zn complex. Note that upon extending conjugation there is a red shift of the Q-band while metallation causes a small blue shift.

(5). Such processes initially generate either an endoperoxide such as seen on the guanine nucleoside base and tryptophan or a hydoperoxide as seen with cholesterol. As an example of the fate of one of these species, cholesterol peroxide can either initiate a massive immunological response [39] cause destruction of the cell [40]or be detoxified [41].

Light and Pharmacokinetics

The crux of PDT is a balance between a drug's distribution and time in a tumor and that of the level and wavelength of irradiation needed to activate that drug. With one exception, 5-aminolevulinic acid (ALA), the drugs currently in clinical trials are introduced into the



Cholesterol (6)

7

Tryptophan (8)

9

Guanine (10)

11

Fig. (5). The photoproduct of singlet oxygen with cholesterol is a hydroperoxide while endoperoxides are generated upon reaction with tryptophan and guanine.

body via intravenous injection. Animal studies have shown that the vehicle is important in the delivery of the drug. The reasons for this seem to lie in knowledge derived from standard cancer therapy. It is believed that it is possible to optimize the delivery of cytotoxic drugs to a tumor by encouraging a low density lipoprotein (LDL) delivery absorption pathway [42]. The ingestion of LDL's by cells is the direct result of receptor based endocytosis into the lysosomes. Degradation disrupts and then releases in excess of 1000 molecule of cholesterols per LDL particle. A demand for high levels of cholesterol has been documented for a number of cancers [43]. Interest in LDL delivery of photosensitizer drugs has been reviewed by Maziére et al. [44] PHOTOFRIN® and its less pure relative HpD have high affinities for neoplastic tissue. It is noted that the adrenals, liver and kidney also possess a high level of LDL receptors and that PHOTOFRIN® also accumulates in these organs. However, PHOTOFRIN® is a mixture of many compounds and to further complicate the issue it is aggregated in the injection solution [45]. Other drugs are delivered in vehicles ranging from Chremophore = EL emulsions to a variety of liposomes for the hydrophobic compounds to aqueous solutions for others (including PHOTOFRIN®). Pharmacokinetic data generally finds that the principal site of adsorption is the liver, bladder, and spleen [46].

The phototoxic drugs currently in clinical trials have relatively low inherent toxicity in the absence of light and the ratio between the tumor, muscle and skin can be changed by modification of the formulation [47]. Another approach to this can be accomplished by modification of the periphery of the chromaphore as described by van Lier's group [48]. Finally, an innovative method yet impractical in the clinical setting is to complex the drug to LDL's [49]. There are no hard and fast rules to these optimization techniques. It would be practical to generate information about a drug from an animal model with serum components that resemble human counterparts. These should consist of tumor/tissue ratios and also included in such studies should be serum distribution levels along with serum component affinities for the drug [50]. These later data are relatively easy to generate because of the fluorescent nature of the photosensitizing drugs. The former data may now be obtained simply by reflective absorption or fluorescent spectrometry [51]. This alleviates some of the tedium, and dangers of modification to the drug's structure due to exhaustive or harsh extraction conditions.

What may be considered a limitation in the field of PDT is the choice by many of the mouse as an animal model where the concentration of LDL's are low [52]. Further complications are generated by choosing a number of different types of mice and the utilization of a number of tumor cell lines for study. Some of these cancer cell lines have been determined to have LDL receptors while this information has not been determined for others. The variability between murine strains is best demonstrated by a paper from Gomer's group [53]. They investigated the effect of relatively lethal doses of PHOTOFRIN II in combination with light in a series of 8 different mice strains. The lethality ranged from10% for $B_{10}D_2$/NSN to 100% for BALB/c. They could modulate these results on the strain C57BL/6 by the addition of drugs such as aspirin and antihistamines, wherein, they observed an increase in survival from 89% for no antihistamine addition to 35% for the addition of an antihistamine. The results from the addition of antihistamines differ from those of Lipson. However, Lipson generally approached drug and irradiation protocols utilizing total lethality of all animals as the endpoint.

Since the murine models may be inadequate, the most consistent experimental animal for assaying the new second generation drugs has been the human. This model will be reviewed in due course. However, there are two other models which would seem to be appropriate. The first is the golden hamster [54], as mentioned above, and the second is a transgenic mouse in which the human tendency towards high levels of LDL's has been inserted [55].

Once the drug reaches the compartments of the tumor its distribution is not well defined. It is known that some PDT drugs have reasonable affinity for these tissues in that excised cells upon irradiation after *in-vivo* injection still results in cell death [46]. Tumors are highly vascularized organs and one property of many effective photosensitizers is their ability to bind to tissues with highly evolved reticulo-endothelium. Inclusive in these types of tissues are the Kupper cells, fixed macrophages and red pulp macrophages of the spleen [56].

At some point the tumor is irradiated with light, Fig. (**6**). Generally, this irradiation will last from 15-30 minutes and will have an energy of 150-300 joules [57]. Light sources these days are more varied than in the days of Lipson. The region of ~670-810 nm is best covered by diode solid state lasers [58]. Drugs which absorb in this region are the naphthalocyanines and the bactereochlorins, along with a class of compounds called the expanded porphyrins, Fig. (**7**). Irradiation for wavelengths which include ~600 nm to 700+ nm occurs with relatively expensive argon pumped dye laser but solid state lasers are becoming available in this region [59]. Compounds which absorb in this region include PHOTOFRIN® (**1**, **2**, **3**, **4**, **5**), BPDMA (verteporfin) (**13**), Sn Etiopurpurin (**19**), Zn Phthalocyanines (**14**), Texaphyrin (**15**) and MACE (**18**), Figs. (**1**)+(**7**). In the case of PHOTOFRIN®,

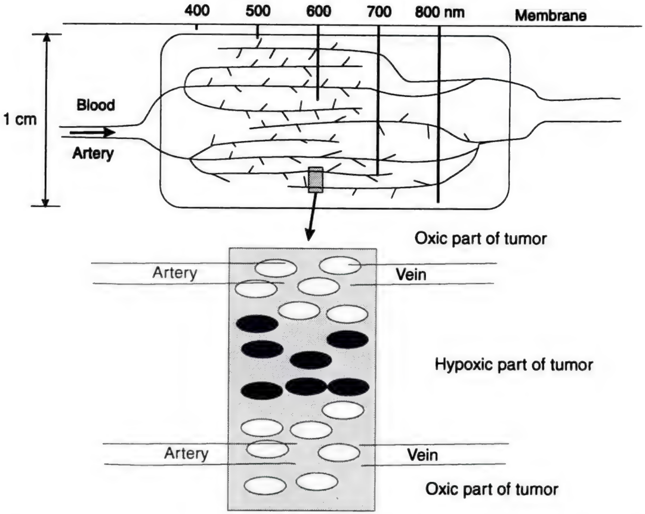
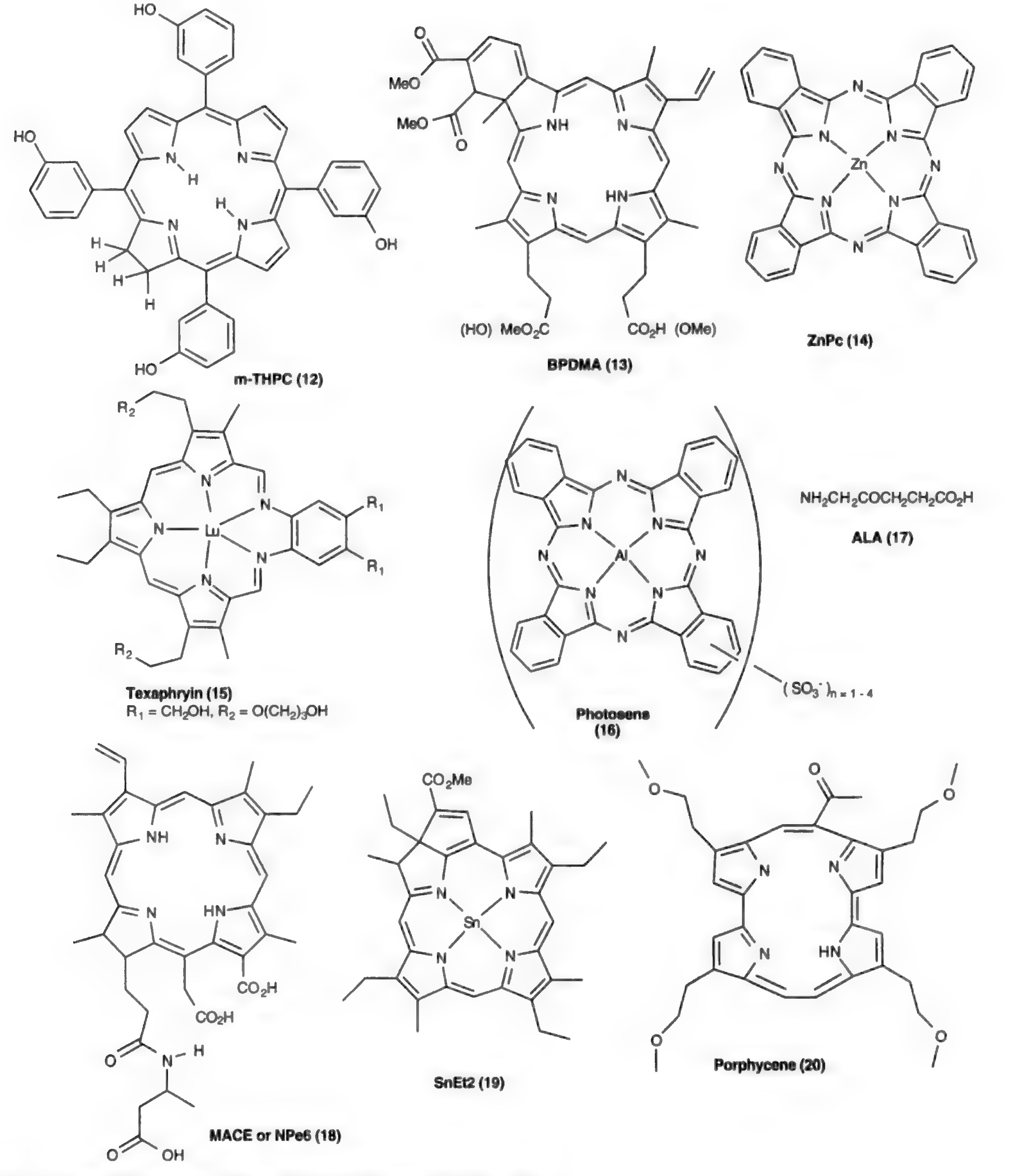


Fig. (6). The three necessary element for PDT are drug, light and oxygen. The figure shows the effective depth of penetration of light at various wavelenghts while the circulation brings drug into the field of treatment via the circulation. An expansion of a small section of the circulation shows that some areas of the tumor may not aquire both drug and oxygen.

Laserscope has developed a modification attachment to YAG lasers already in the hospital setting which consists of freqency doubling coupled to a dye laser [60]. Pulsed lasers such as gold vapor lasers, copper vapor lasers and eximer pumped laser dye lasers also have been applied to PDT. These may suffer a disadvantage, due to their short duration but high intensity, of prematurely bleaching the drug by further pumping energy into the already excited state of the drug and also damage to their light delivery fibres can also occur.

Irradiation of the tumor initiates a cascade of responses during a short time frame. Oxygen is necessary for this cascade as determined by experiments in which the tumor is clamped rendering it hypoxic [61]. Under these conditions the drug PHOTOFRIN® is ineffective in causing tumor necrosis. Primary toxicity to all the cells in the tumor, when oxic, is perhaps only 20-30% for PHOTOFRIN® [46]. The event which may best describes direct cellular killing is the disruption of the Ca^{2+} channels. This type of process is known to cause cell death [62]. It has been shown that PDT with PHOTOFRIN® can cause a large increase in Ca^{2+} concentration in the cell [63]. The increase in the concentration of this ion is perhaps linked to plasma serum leakage. Short irradiation times results in only a transient effect. Longer irradiation is totally disruptive to the internal Ca^{2+} transport system.

With specific compounds derived from tetraphenylporphyrin photosensitizers, a rather unusual process may also account for the cell death. These compounds can bind to the microtubules of a cell, at least *in-vitro*. Irradiation of such a complex of drug connected to the spindle apparatus would



m-THPC (12)

BPDMA (13)

ZnPc (14)

Texaphryin (15)
$R_1 = CH_2OH$, $R_2 = O(CH_2)_3OH$

Photosens (16)

$NH_2CH_2COCH_2CH_2CO_2H$

ALA (17)

MACE or NPe6 (18)

SnEt2 (19)

Porphycene (20)

Fig. (7). Second generation drugs which are presently in clinical trials.

obviously cause problems in cell cycling. This process is under investigation by a number of groups [64,65]. However, it is unlikely that all photosensitizers have such an affinity for this subcellular system in that they have quite large structural differences.

Direct cell kill cannot be the only major source of disruption to the tumor. The vasculature of the tumor may also be affected by PDT. Several observations support this conclusion. Despite extensive irradiation some cells in the hypoxic region of the tumor survive

[66]. This was determined by excising the tumor and examining the growth pattern of the residual cells. Since hypoxic cells express the largest level of Ras, the expectation would be that the remainder of the tumor would be very active in metathesis [67-68]. This is not the case with PDT. In the case of PHOTOFRIN® the vasculature is disrupted almost immediately. This is followed by complete shutdown of the blood supply imparting, after treatment, total hypoxia. Such a situation as clamping the blood supply to a tumor is known to cause complete tumor death within a day. Other photosensitizers are known to follow a similar pattern of partial direct kill of the clonogenic cells followed by vascular shutdown. The importance of regional vascular shutdown was reported by Fingar et al. [66] in which tissue directly adjacent to the tumor was shielded from light. This reduced regional dose seemed to allow angiogenisis through the nearby viable cells to resupply the tumor with nutrients.

The events which cause extensive vasculature damage may be the result of localized signal transduction. These 'natural' immunogenic responses, as with vasculature shutdown, seem to be similar for most sensitizers which have been investigated. As an example, in the human squamous carcinoma, PHOTOFRIN® is known to cause the release of PGE_2, $IL_{1\alpha}$ and β, IL3, IL6, $TGF_{\beta1}$, INF_{γ}, GMCSF and AA [69-70]. These species are also released by stimulated macrophages. Normal human endothelial cells were found to release 6-Keto-$PGF_{1\alpha}$ along with $PGF_{2\alpha}$ and AA upon PDT. Also of note, as activating species released upon PDT treatment, are histamine, serotonin [72] and TNF_{α} [73]. Inhibitors of the release of these amines, such as with indomethacine, resulted in the moderation of the PDT effect [74]. This was also shown to occur when animals genetically deficient in mast cells were treated with PDT [75]. As an example of a human *in-vivo* experience with such species, the cytokines TNF, and both IL-2 and IL-1 were released by patients undergoing treatment for bladder cancer [76].

The nature of the increase in PDT induced tumor cell killing by the microvasculature has been discussed above. A further potentiation may be as a result of macrophage stimulation by cells undergoing PDT. Korbelik and Krost [77] suggested that 'inflamed' tumor cells release alkylglycerols, lysophospholipids and alkyllysophospholipids. These species are known to be potent macrophage stimulators. They observed a significant increase in tumor cell toxicity (murine squamous) in the presence of macrophages as compared to normal cells (normal murine kidney). A similar activation of macrophages was discussed by Yamamoto et al [78]. In animals injected with only 300 ng of PHOTOFRIN® the response to ambient laboratory light was to induce Rc-receptor-mediated phagocytic response five fold while superoxide generating capacities of peritoneal macrophages were upregulated by a factor of 7. In a related topic the sensitivity of splenic lymphocytes stimulated by the mitogen concanavalen A showed that uptake of drug increased by a factor of ~4 which resulted in an increase in phototoxicity of a factor of ~10. A similar observation by Franco et al was the suppression of murine spleen cells treated with HpD and light [79]. Clearly, the state of the tumor cell and the circulating immunological cells are factors in the extent of cell death.

These responses to PDT stimulation may not be all that specific. They resemble actions of cells also placed under various other types of stress, including UVB-light, UVA-light with psoralens and oxidative stress. For instance UV light is known to release TNF_{α}, along with IL1 and histamine. The type and number of cytokines derived from keratinocytes upon activation by UVB have been exhaustively studied [80], and include, along with those discussed above, IL8 and IL10 along with colony stimulating factor, and transforming growth factor. A recent paper showed that extremely small amounts of light (UVB) can begin the release of a wide array of protein kinases in skin [81]. The field of PDT is too new for all the factors released upon mild irradiation to have been elucidated. However, PUVA (psoralen/UVA) light therapy seems also to follow this general pattern of cytokine activation [82]. Historically, the process of DNA crosslinking has been suggested as the mechanism for this drug-light treatment. However, more recently there is evidence that results of PUVA therapy may, in addition, be a response to oxidative stress and even singlet oxygen generation [83].

An experiment by Gruner et al [84] in 1985 best illustrates the similarity between immune responses between PDT (HpD derived), UV and UV with psoralens. It was found that the survival of allogenic skin grafts treated under PDT conditions increased due to treatment with either UV-light, UV-light and psoralens or visible light and HpD. Also observed by the authors was the inhibition of murine antigen presenting cells. This work was reproduced at QLT PhotoTherapeutics utilizing BPDMA. The paper by Lynch et al. [74], an extension of the work by Elmets and Bowen [85], sheds some light on this immunosupression. The authors investigated the nature of subtoxic peritoneal photodynamic (PPDT) treatment of mice which causes the loss of contact hypersensitivity (CH). After such treatment the response to exposure to the irritant 2,4-dinitrofluorobenzene (DNFB) in a footpad swelling assay was markedly reduced as compared to controls. Such suppression is adoptively transferable as demonstrated by the injection of splenic lymphoid cells from a group of mice exposed to PPDT and contact irritant into normal mice. These transplanted mice showed a 78% suppression of the CH response as compared to controls. The PPDT treated mice in another experiment did not recover CH response upon the transplantation of normal spleen cells. Such adoptive CH was not limited to the original irritant as demonstrated by changing the challenge on the transplanted mice to oxazalone where the CH effect was maintained. The transplantation of just PPDT treated mice spleen cells did not show CH suppression, indicating that an initial antigen challenge was necessary. When cells from PPDT treated animals were utilized as stimulators in a one-way mixed lymphocyte reaction (MLR), there was a 90% decrease in proliferation. Separation of cell populations of spleen cells from mice which had undergone PPDT along with sensitization with DNFB into a non-adherent T-cell enriched fraction and a adherent B-cell/macrophage fraction (MO) were separately transplanted into mice. Only animals which received the B-cell/MO cells were found to be suppressed upon challenge with DNFB. A further such experiment, showed that purification of just the MO fraction of cells and then transplantation into mice followed by a challenge also resulted in CH suppression. PPDT, also showed an increase in the levels of neutrophils and lymphocytes along with an increase in serum amyloid concentrations on the subject mice [86]. The CH suppression could itself be suppressed by the implanting of an indomethacin pellet prior to treatment with PPDT and irradiation.

The primary toxicity to cells in PDT is quickly followed by changes in the nature of the vasculature surrounding the tumor along with extensive localized immunological changes. Most recently these have been categorized under the umbrella of programed cell death (apotosis) [87]. Obviously, these types of cell responses to PDT stimulation add to the complexity when assaying a new photodynamic drug let alone when attempting to correlate structure activity relationships (SAR). However, capitalizing on these post direct irradiation properties has led to some of the most promising areas of treatment for human diseases. These range from autoimmune diseases to ones involving the vasculature.

Stucture Activity Relationships

Introduction

The evaluation of a photosensitizer as a human phototherapeutic agent takes a course through simple *in-vitro* assays to more sophisticated *in-vivo* assays. There are many compounds under evaluation as second generation sensitizers. As mentioned above there is often no consistency in their evaluation, even among the same groups. Therefore, any section evaluating SAR can only survey selective data which is internally consistent. Such a sequence should give the reader a roadmap, if they ever intend to develop a photosensitizer. For a more extensive evaluation of SAR, a review from our group has just been submitted for publication [88].

Serum Distribution

As previously mentioned, most researchers in the field believe that a profile of a drug in the serum is a necessary first step towards a SAR evaluation. It is our belief that this sort of evaluation should also include the dimensions of this binding. Such an affinity number might be helpful in that several compounds developed for clinical trial have had such a strong interaction with human serum albumin (HSA) or other tissues, that they take weeks to clear from the system. A mini-review by Kongshaug is an example of such a systematic survey in this area [89]. These types of investigations are not exotic but should be done prior to that of any of the other assays.

Various methods are utilized to determine the distribution of photosensitizers in human serum. These include ultracentrifugation, aqueous gel permeation chromatography and gel electrophoresis. The later two suffer from problems associated with either aggregation of the photosensitizer or poor resolution between serum components. Affinities between a drug and a serum component are measured only with serum albumin. This may be a result of the ready availability and high concentration of this material in serum (HSA - 40-50g/mL, gamma-globulin - 8-18g/mL, HDL - 1.3 g/mL, LDL - 0.8 g/mL) [90]. It cannot be emphasized enough that porphyrins-like compounds aggregate. Close inspection of the efficiency of the fluorescence as compared to the absorption spectra can give one some indication of this. Without this, the distributions could reflect only the distribution of aggregates in serum components. New methods to assay affinities between a protein and a pharmaceutical entity have been developed by the groups of Walsh [91] and Whitesides [92] which give extremely accurate numbers utilizing capillary electrophoresis. This method has yet to be applied to PDT agents. It may also be possible to find distributions of drugs in serum components with this analytical technique, since injection sample sizes generally are 10 nanoliter and solvent usage is only a few microliters per run.

The affinity of dipropionic acid porphyrins for HSA can be extremely high. Table 1 gives a brief summary of the ranges expected for various simple porphyrins [93]. The affinities have been seen to vary greatly even when assaying the same compound. For instance the results for protoporphyrin range from 0.5 to 3200 [93]. HSA is also known to have two distinct sites of affinity for porphyrins. A thorough investigation should try to determine whether both sites are involved in binding. Finally, close inspection of tryptophan fluorescence quenching by the photosensitizer may give some indication of the type of binding being observed.

Table 1. Selected Porphyrin Affinities to Human Serum Albumin [93]

Porphyrin	Affinity to HSA K = x 10^{-6}
Hematoporphyrin	~ 2.0 ± 1.5
Hematoporphyrin Zn(II)	4.7
Hematoporphyrin Fe(III)	3.2
Protoporphyrin	~0.5-3200
Protoporphyrin Sn(IV)	0.21
Protoporphyrin Fe(III)	0.5

A survey of the work of Kongshaug et al on the distribution of a series of derivatives of various esterified hematoporphyrins are compared in Table 2. The distribution of Hp was principally into HDL and heavy proteins (albumins) [94]. As the lipophilicity of the derivative increased, the levels in the LDL seemed to increase at the expense of the levels in the heavy proteins. Another series of compounds that were tabulated in the review were the sulfonated tetraphenylporphyrins (TPP). Moving from the tetrasulfonated TPP's to the mono-sulfonated TPP's showed a similar trend towards increased LDL binding. A note of caution should be expressed about the work in that neither the derivatives of hematoporphryin (mixture of diasterioisomers and enantiomers) and the sulfonated TPP's (mixture of regioisomers) were pure compounds.

Table 2. A Series of Hematoporphyrins and Their Serum Component Distribution [94]

Photosensitizer	LDL	HDL	Heavy proteins
Hp	8.6	62.4	17.7
Hp-dimethyl	20.5	65	13
Hp-diethyl	20	63	17
Hp-dipropyl	23	63.9	13.2
Hp-dibutyl	23	65	12
Hp-diamyl	21	66.1	13
Hp-dioctyl	27.1	66.2	6.8
Hp-dicyclohexyl	19.7	62.2	18.2

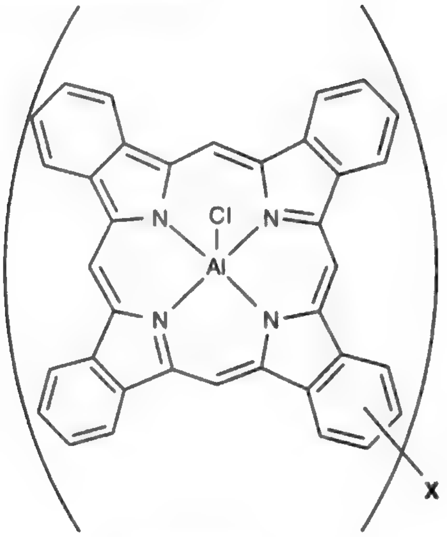
Photophysics

Modification of the periphery of a photosensitizer should have little to do with their ability to produce singlet oxygen. However, a change in the chromophore can have a dramatic effect. Spikes and Bommer [95] showed that metallation of mono-aspartylchlorin e_6 (MACE or NPe6) (**18**) changed the quantum rates of photobleaching. The unmetallated material gave a quantum yield of photobleaching of 8.2 x 10^{-4} while the Sn and Zn derivative gave values of 5.7 x 10^{-6} and 1.9 x 10^{-2}, respectively. This greater than 1000 difference is significant because it relates the drug to its stability under light flux and therefore, its phototoxicity. These numbers were generated in neat buffer. The addition of serum would normally increase the rates of photobleaching because protein components act as a reservoir for electron transfer reactions. Removal of the aspartyl group had some effect on the rates of photobleaching. However, these were much less dramatic than shown for the metals. Also, depending on the solvent, rates can be significantly different. For NPe6 with a given rate of bleaching in water of 1.00, the rate decreases to 0.10 in dimethylformamide and 0.035 in ethanol despite a concurrent increase in the level of oxygen in these solvents. It is known that the bleaching rates of porphyrins increase as the dielectric constant of the solvents increase [96]. This may, perhaps, relate to the possible difference in photobleaching of compounds in different subcellular compartments.

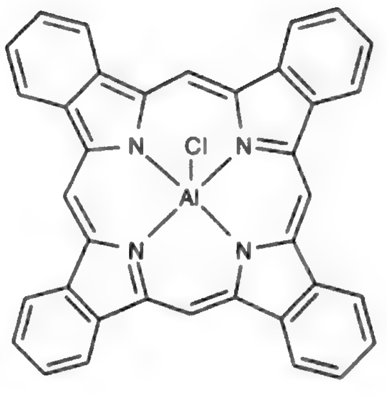
In-Vivo Phototoxicity

The comparison of various drugs by *in-vivo* and *in-vitro* assay is difficult because of variation between cell lines, light dosages, formulations, and determination of cell survival used by each research group. This can be mitigated by following a single series of experiments. In work by Margoron et al. [97] a series of various substituted aluminum phthalocyanines were investigated for their activity *in-vitro*. The polarity of these compounds decreased from the very polar tetrasulfonated compound to the tetra-iodonated compound shown in Fig. (**8**). Unique to this study was the utilization of radioactive labeled drugs. This allowed accurate cellular uptake information without utilizing fluorescence which might be confounded by environmental quenching.

In a EMT-6 cell line, all the compounds were found to continue to take up drug over a twenty four hour period. A general trend which followed partition coefficients between a octanol/tris buffer system was observed. The more hydrophobic dyes tended to incorporate faster into cells. Dye release back to the media seemed not to be dependent upon



21. X = $(SO_3^-)_4$
22. X = $(SO_3^-)_3$
23. X = $(SO_3^-)_2$
24. X = $(SO_3^-)_1$



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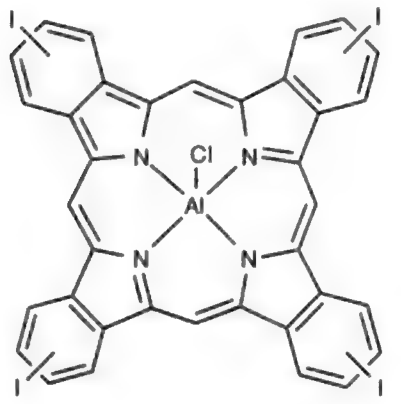

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Fig. (8). Aluminum phthalocyanines are shown with either 4, 3, 2, 1, or 0 sulfonic acid groups along with one with 4 iodo atoms.

hydrophobicity. Phototoxicity showed a trend in that the level of least sulfonation produced the greatest phototoxicity (**21**-**25**). The tetra-iodo (**26**) compound was rather poorly phototoxic despite having displayed good cellular uptake. Although it does seem to be taken into the cellular membranes, the presumed site of toxicity, this material had a high tendency towards aggregation. This resulted in poor phototoxicity due to quenching of the excited state. Other structure activity correlations have sometimes showed such a lipophilicity trend [98], while others have not [99].

In-Vivo Distribution

Two areas should be considered when observing the distribution of a drug. The first is the structure (hydrophobicity, size, polarity) of the drug and second is the formulation in which it is delivered. Comparison of the effect of formulation was described by Jori's group [100], in which Zn phthalocyanine (**14**) formulated in either a DPPC/cholesterol liposome or just DPPC liposomes, was administered to mice. They found that the formulation which contained cholesterol, which encourages delivery to the LDL receptors, increased the concentration in the tumor from 0.28 to 0.42 micrograms per gram of tissue.

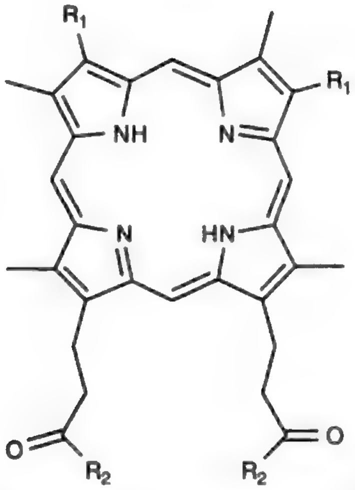
In an extensive paper by Woodburn et al. [99] the distribution of the compounds shown in Fig. (**9**), in mice carrying a C_6 glioma tumor, were evaluated at 6 and 24 hours. Of course the drug concentration in the tumor was important for therapy as is a high level in tumor relative to those in the skin and muscle. The zwitterionic species (**35**) and the cation (**36**) were found to be the compounds with greatest uptake and relative distributions. A general explanation was offered for this uptake preference. Compound **35** seemed to prefer concentration in the lysosome while compound **36** seems to prefer the mitochondria as is generally found for such cationic dyes [101]. This subcellular distribution was determined by the utilization of confocal laser scanning microscopy [102]. A technique which must be viewed with caution when applyied to dyes which would have different fluorescent quantum yields in different types of tissues. It is also worth noting that phototoxic cationic dyes are susceptible to multidrug resistance, so may not form the basis of a photosensitizer which could be continually reused [103].

Table 3. Four Hematoporphyrin Analogues and Their Distributions in Selected Tissue [99]

	Time	Tumor	Skin	Muscle	Blood
Hp	3 hrs.	0.51	2.87	0.57	1.35
	24hrs.	0.43	0.40	0.22	0.24
HpD		10.2	4.31	2.85	3.00
		6.20	4.20	1.17	1.94
Hp-11(35)		11.7	3.20	1.98	11.7
		7.20	1.00	1.72	4.45
Hp-12 (36)		2.53	5.10	2.81	1.80
		8.23	3.26	1.46	2.28

1. Concentration in micrograms/gram of wet tissue
2. Standard deviation ~10% for most values
3. Each point mean of 9 measurements

Table 3 describes concentrations in tumor, serum, skin and muscle of a series of compounds. The samples were injected as an aqueous solution intravenously at 20 mg/Kg (~mouse 20g, C_6 glioma



COMPOUND	R_1	R_2
1	$HC(CH_3)OH$	OH
27	$HC(CH_3)OCH_3$	OCH_3
28	$HC(CH_3)SCH_2CH(NH_2)CO_2H$	OH
29	$CHCH_2$	$NH(CH_2)_2N(CH_3)_2$
30	$CHCH_2$	$NH(CH_2)_2N(CH_2CH_2)_2O$
31	$CH(CH_3)OCH_2CH_3$	OH
32	$CH(CH_3)OCH_2CH_3$	$NH(CH_2)_2N(CH_3)_2$
33	$CH(CH_3)O(CH_2)_2OCH_2CH_3$	OH
34	$CH(CH_3)O(CH_2)_2OCH_2CH_3$	$NH(CH_2)_2N(CH_3)_2$
35	$CH(CH_3)S(CH_2)_2N(CH_2CH_2)_2O$	OH
36	$CH(CH_3)S(CH_2)_2N(CH_2CH_2)_2O$	$NH(CH_2)_2N(CH_3)_2$
37	$CH(CH_3)SCH_2CO_2H$	OH

Fig. (9). Shown are a number of compounds which have been studied by Woodburn et. al. in a murine/glioma model.

tumor, CBA mice). Tissue was extracted with a methanol/chloroform solution. Some samples which were poorly fluorescent were converted to Hp by acid hydrolysis and heat. Some other examples of tumor/muscle ratios and tumor/skin ratios for well known compounds undergoing clinical trials are listed Table 4.

Table 4. Optional Drug Tumor/Muscle and Tumor/Skin Ratios

	Time[a]	Tumor	Mice	Tumor/ muscle[b]	Tumor/ Skin[b]
ZnPc [175]	24hours	MS-2	BALB/c	5.71	~3.0
BPDMA [176]	3 hours	M1	BALB/c	19.2	2.3
AlPCS4 [177]	24 hours	Colon	BALB/c	28	2
NeP6 [178]	10 hours	BA mam.	C3J/HEJ	10.6	2.2
THPC [179]	24 hours	Colon	Swiss	15	1.1

a These are optimal times for ratio - drug level may not be enough to treat

b Average of four experiments from four researchers.

In-Vivo Treatment

The treatment of mice *in-vivo* is measured by determining the number of tumor free animals at some point post treatment versus a control set. To obtain meaningful numbers more than 10 animals should be assayed for each drug/light/time of treatment series. Therefore, the optimal time of treatment should be established first by either extraction techniques to assay drug level or by reflective spectroscopic techniques utilizing a smaller number of animals for each time point. Without this determination, because of the number of variables, animal usage quickly expands.

Chemistry

The spectra of conjugated tetrapyrrolic macrocyclic compounds distinguish themselves by containing two distinctive peaks [104]. The Soret-band generally lying in the region from 400-500 nm with a molar extinction coefficient of greater than 100,000 and the Q-band which is found at a wavelength beyond 600 nm with an extinction which ranges from 3,000-300,000. The Q-band is the desired region of the spectrum for irradiation because it lies beyond the visible light absorbing compounds in the body and below the region where water begins to absorb at ~800 nm [105,106]. The ability to sensitize and give singlet oxygen is dependent upon the energy of the triplet state of the sensitizer which must be greater that 1266 nm since there is an energy barrier for the triplet energy transfer through the exciplex compelx to triplet oxygen. Compounds which have absorption bands which lie much beyond 800 nm fail to have the energy in their triplet state to readily transfer to ground state oxygen. Fig. (**10**) illustrates the type of spectra generally found for porphyrins (**38**), chlorins (**39**) and bacterochlorins (**40**). Compounds such as chlorins and bacterochlorins are available from either natural sources or by synthetic modification of porphyrins.

The basic chromaphore of a porphyrin can be changed by not only disrupting the conjugation of the perpheral double bonds but, also, by extending the conjugation through extra double bonds (expanded porphyrins, porphocyanines, (**84**), Fig. (**25**), increasing the number of pyrroles in the ring (**15**) (texaphyrin, Fig. (**7**)) or fusing the system onto other aromatic systems (phthalocyanines and naphthalocyanines, (**16+18**) Fig. (**24**)). It is not in the domain of this review to describe all the chemistries involved in the formation of these types of heterocycles. This area is under currently under review by our group [107] Considering the difficulty of fully evaluating a compound as a pre-clinical agent the number of compounds and their simple derivatives seem excessive. However, limitations placed upon the chemistries leading to and the stabilities of these second generation compounds somewhat simplifies the choice. Further culling of the field of compounds is achieved by loss of stability as the conjugated system is extended or by their difficulty in formulation. The more extended chromaphores appear much more sensitive to destruction by singlet oxygen and other photobleaching mechanisms. Availability or yield limits some of the derivatives of natural products or the more exotic macrocycles. The present drugs in clinical trials with one notable exception (texaphyrin) are ones whose chemistries are straightforward and based in the 1960's and earlier.

All of the compounds mentioned above can be metallated. The result of such a process often results in a shift of the chromaphore to the blue, Fig. (**4**). Since addition of a metal adds a level of symmetry to the chromaphore, the number of bands in the optical absorption spectrum usually simplifies. Paramagnetic metals (Fe^{+3}, Cu^{+2}) can also have the effect of quenching the lifetime of the triplet by a factor of sometimes greater than 1000. Since it is this state which is responsible for the energy transfer to ground state oxygen, the reduction in singlet oxygen quantum yield is substantial. The result of paramagnetic metallation is a predominance of photodestruction through type I photoprocesses and little or no singlet oxygen formation.

5-Aminolevulinic Acid (ALA)

One of the most investigated compounds in PDT clinical trials today possesses no chromaphore. In the mid 1980's Rebiez et al [108] at Michigan State proposed that 5-aminolevulinic acid (ALA) could be utilized as a larval insecticide. They recognized at this stage of development insects produced

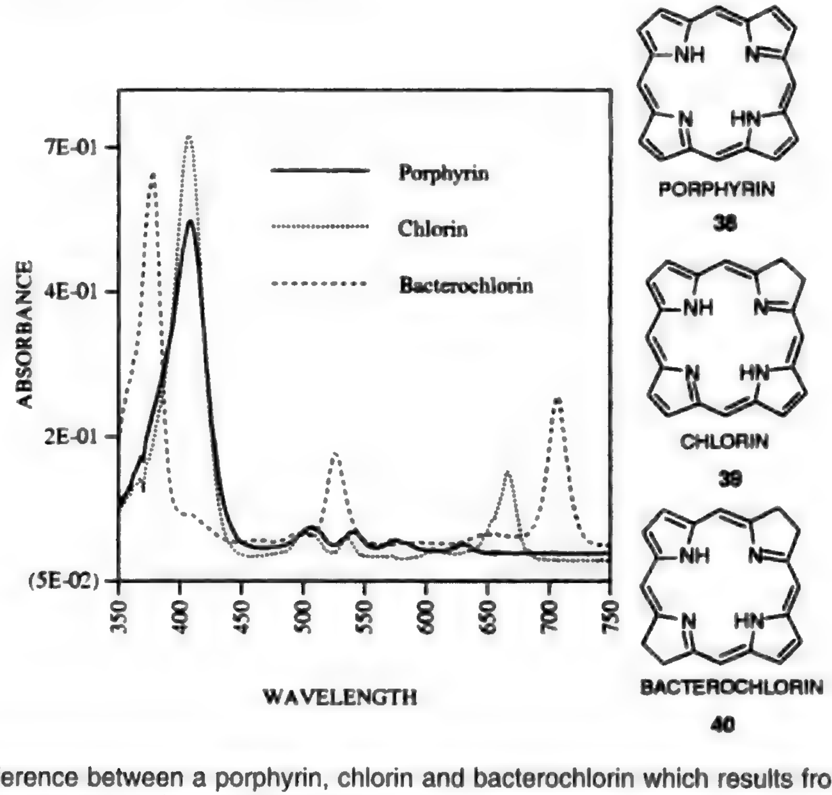


Fig. (10). Shown is the difference between a porphyrin, chlorin and bacterochlorin which results from the level of reduction of the beta-double bond and the spectrum of each of these species.

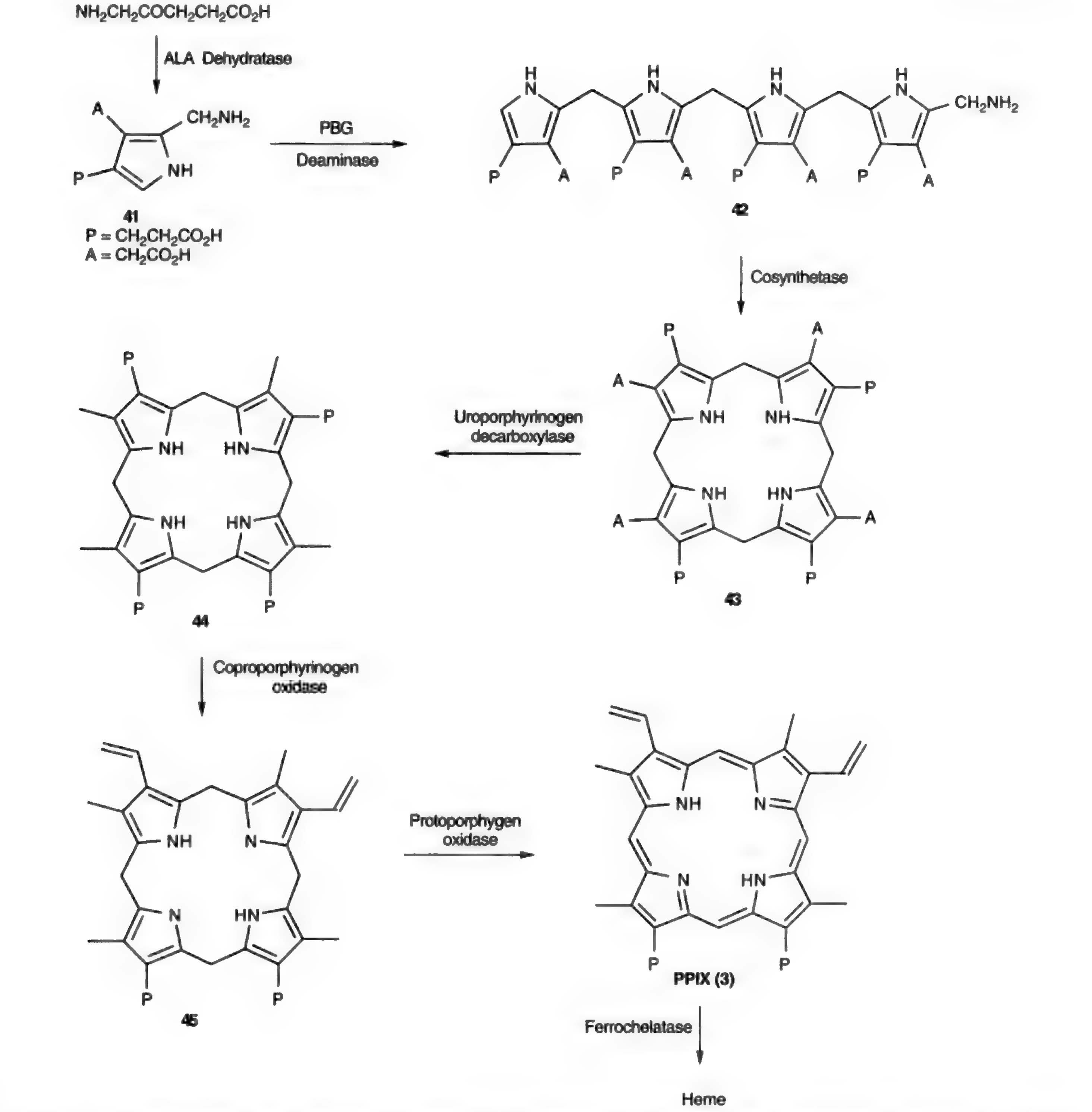
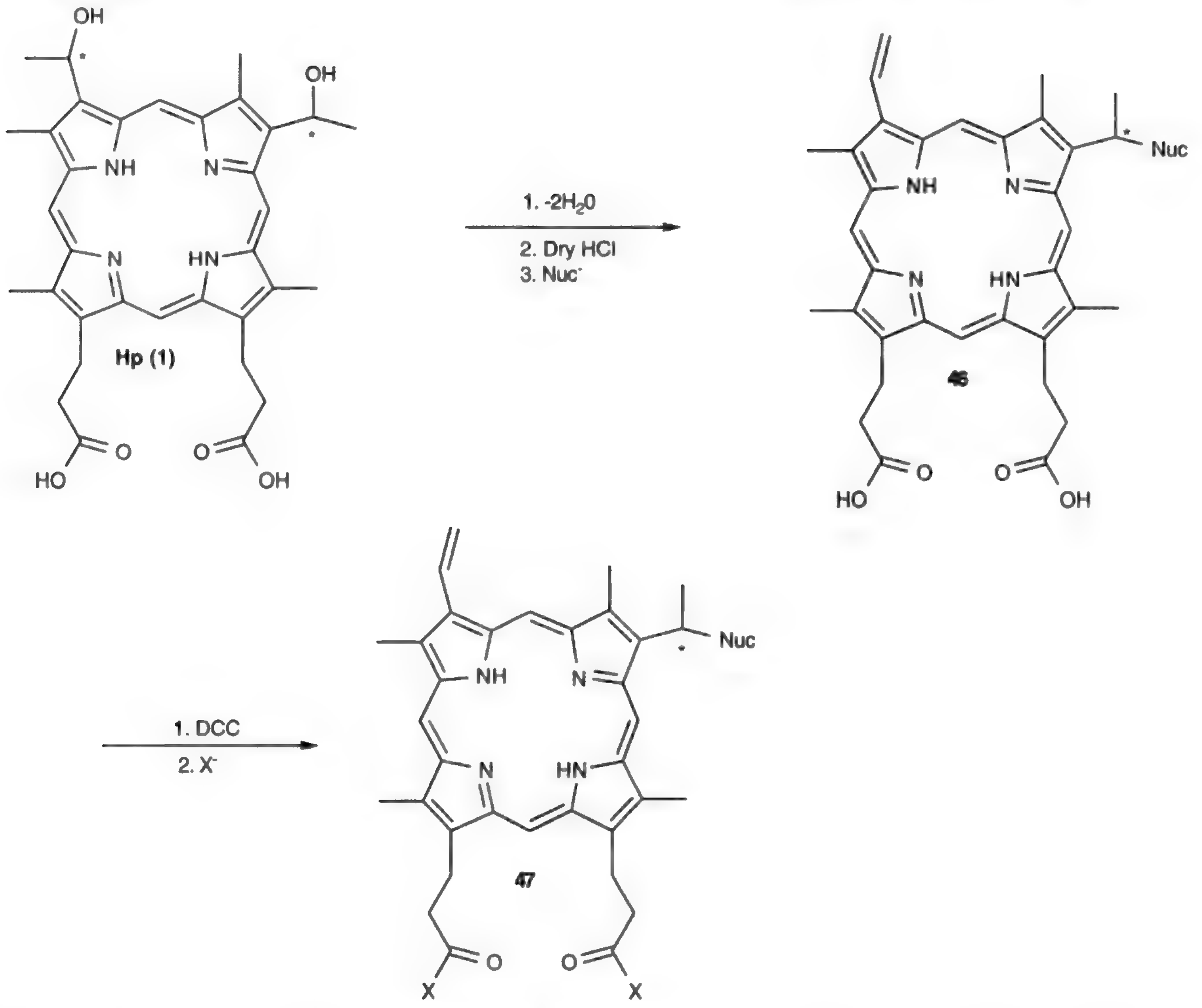

NH2CH2COCH2CH2CO2H
ALA Dehydratase
A
CH2NH2
P
NH
41
P = CH2CH2CO2H
A = CH2CO2H
PBG
Deaminase
H N
CH2NH2
P A P A P A P A
42
Cosynthetase
43
Uroporphyrinogen decarboxylase
44
Coproporphyrinogen oxidase
45
Protoporphygen oxidase
PPIX (3)
Ferrochelatase
Heme

Fig. (11). The aminolevulinic acid cascade to produce PPIX consists of a number of enzymatic steps. Metallation of PPIX to heme occurs at a different subcellular site.

protopoprhyrin IX (**3**, PPIX) at a level higher than the subsequent metallation to heme. Pottier et al [109] also in the early 1980's recorded contrasting intensities of PPIX fluorescence in different tissues after the administration of ALA. This 'ultimate' pro-drug utilizes a cells inherent ability to make PPIX via a cascade of enzymatic processes outlined in Fig. (**11**). The final process to metallate this porphyrin via ferrochelatase seems to be somewhat deficient in tumor cells [110]. This balance results in the accumulation of the phototoxin *within* the cell as opposed to other types of drugs mentioned earlier.

Addition of exogenous ALA either topically or systemically is quickly followed by the production of PPIX [111]. It seems the success of the topical application is based upon the observation that damaged skin is more permeable to penetration of this drug than that of normal skin. The behavior of the orally active drug is interesting in that, under a pharmacokinetic multi-compartment analysis, the



X = can be amine, alchohol or thiol

Fig. (12). Shown are standard reactions applied to either Hp or PPIX by which analogues can be readily generated.

production of active drug is dependent upon passing through the stomach into the circulation followed by the microvasculature, through a cell membrane into the subcelluar regions which are responsible for heme synthesis followed finally by converstion to drug. In the mitochondria a specific benzdiazepine receptor [112] which has a high affinity for PPIX might be responsible for a selective site damage by this photosensitizer. Other simple derivatives of di-propionic acid porphyrins and chlorins may mimic such uptake into this receptor.

Derivatives of Natural Porphyrins and Chlorins

Earlier in this review we discussed the production of HpD and PHOTOFRIN® from hematoporphyrin (Hp, **1**). Only relatively recently have simple derivatives of this readily available porphyrin been produced for investigations into their SAR [113]. Dehydration of the alpha-hydroxyl group can be readily achieved by heating the compound in DMF. Activation of the vinyl group with HCl followed by addition of a nucleophile results in either ether, amino or thioether derivatives, Fig. (**12**). Derivatization of the propionic acid side chains of Hp is possible through standard activation utilizing reagents such as dicylclohexyl carbodimide (DCC) to activate the acid followed by addition of an amine, alcohol or thiol, Fig. (**12**).

An example of how the chromaphore of PPIX can be directly modified is found in the work of in our group [114] which was based upon earlier work of Johnson's group [115]. It was observed that a Diels-Alder reaction was possible between the inherant diene of PPIX and various 'strong' dienophiles, such as tetracyanoethylene and dimethylacetylene-dicarboxylate, Fig. (**13**) [116]. This reaction resulted in the formation of a chlorin like species with its Q-band shifted from 632 nm (exinction ~3,000) to 666 nm (extinction ~50,000). A further shift in the chromaphore to 690 nm (extinction ~35,000) was noted upon treatment with strong base such as DBU. It was recognized and derivatives were generated that showed the capability of modification at

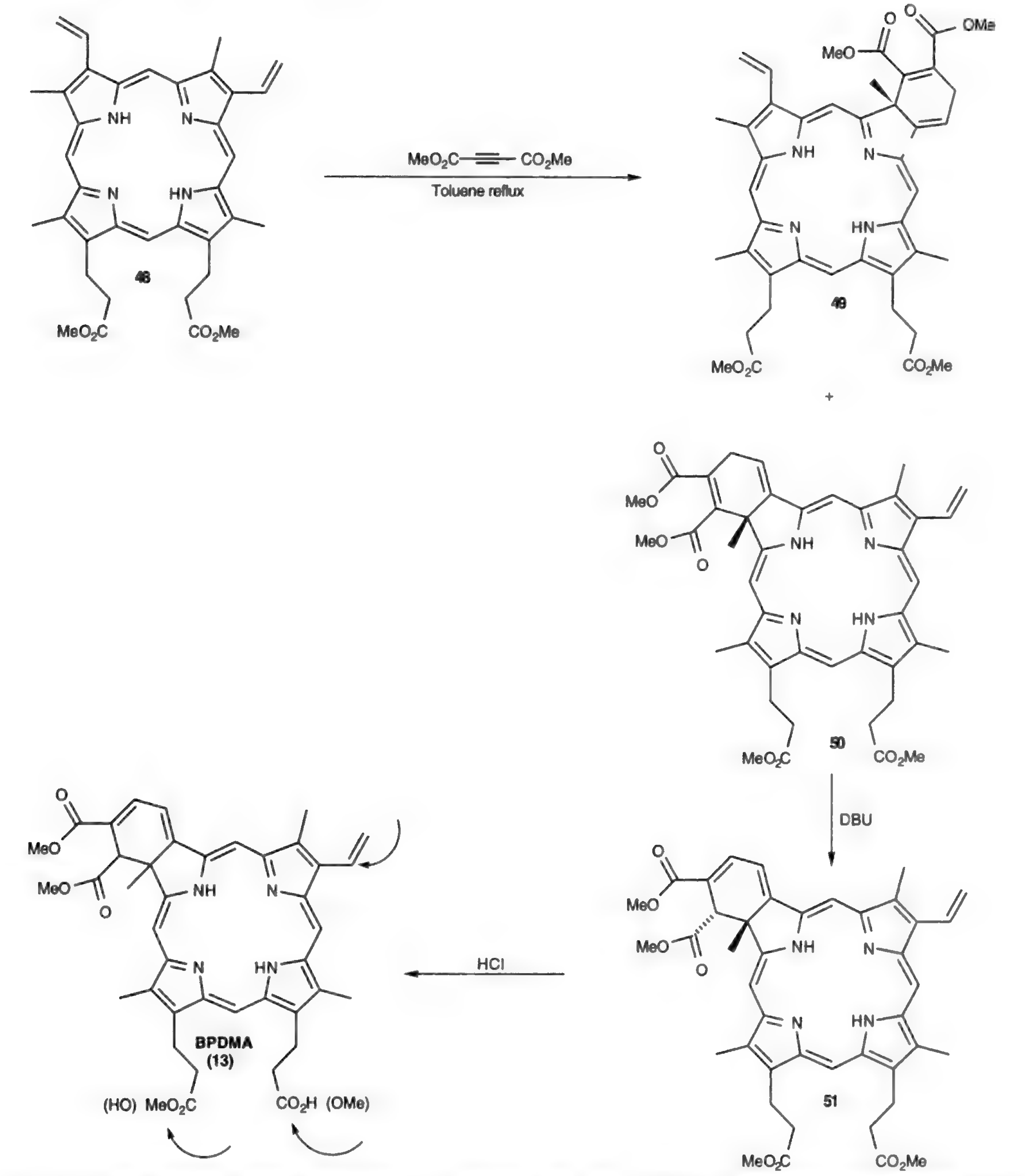


Fig. (13). These benzoporphyrin derivatives are produced upon the reaction of the dimethylester of PPIX with dimethylacetylene dicarboxylate. Arrows show sites which are easily modified.

the vinylgroup along with the peripheral methylester groups [117-120]. During initial investigations the mixture of regioisomeric monoacids of either the B-ring or A-ring derivative showed promising *in-vitro* and *in-vivo* results. Liposome formulation showed that the monoacid A-ring provided the higher drug/lipid ratios and stability [121].

An interesting intramolecular Claisen rearrangement was performed on the mono-hydroxyethyl derivative

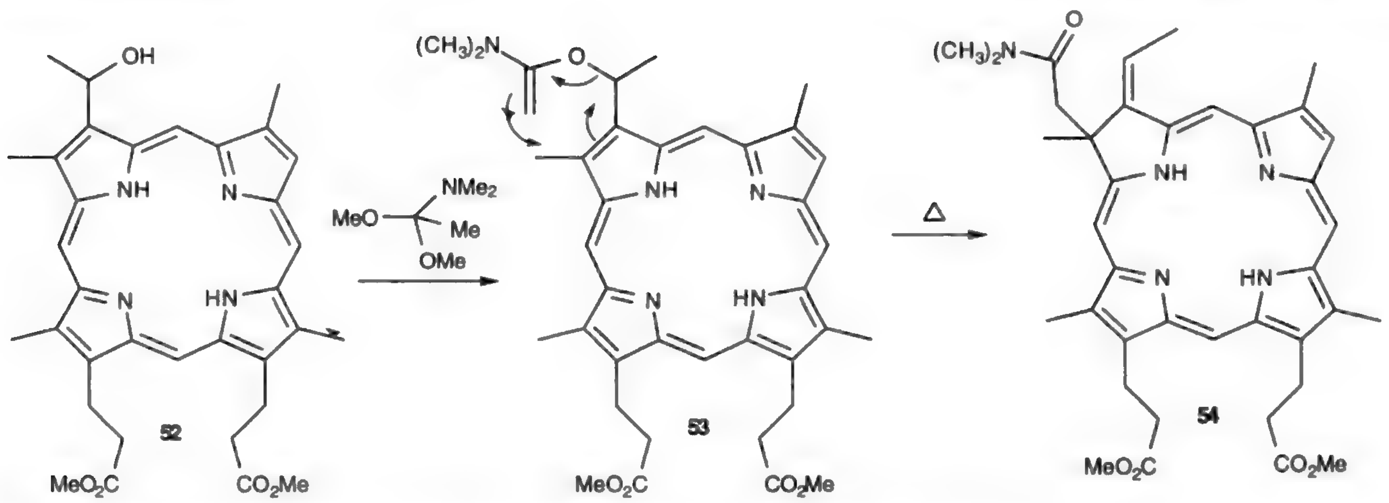


Fig. (14). Interesting intramolecular electrocylic reaction results in a chlorin like molecule.

52 of HvD. Addition of N,N-dimethylacetamide dimethylacetal to this species with heating resulted in the formation of dimethylamide, Fig. (**14**) [122]. A shift of 15 nm upon the reduction of the exocylcic double bond is somewhat surprising and was probably due to the release of strain on the aromatic system. Other derivatives tapping this vein of electrocyclic chemistry have also been published by Montforts and his colleagues [123].

One of two photoproducts of PPIX (**55**) has been used in the synthesis of a rather unique conjugate with thiobarbituric acid, Fig. (**15**) [124]. The resulting shift in the spectral absorption range results in a very broad band centered over 100 nm to the red over that the starting material which extends to ~900 nm. This derivative showed no photodynamic based toxicity in a mouse tumor assay. As mentioned above it is possible to generated a compound which does not have the triplet energy requirements necessary for the production of singlet oxygen. This may be such a case.

One of the earliest second generation phototoxins investigated for clinical trials was mono-L-aspartylchlorin e_6 (NPe_6 or MACE, **18**) [125]. This material is readily created by the addition of di-t-butly-aspartylamine to chlorin e_6 which had been activated with DCC. This results in the formation of both the mono and di adducts, (Fig. (**16**). Chromatographic separation which is followed by deprotection with trifluoracetic acid gives the tetracid product (**18**) and pentacid (**58**). This is the only fully water soluble compound undergoing clinical

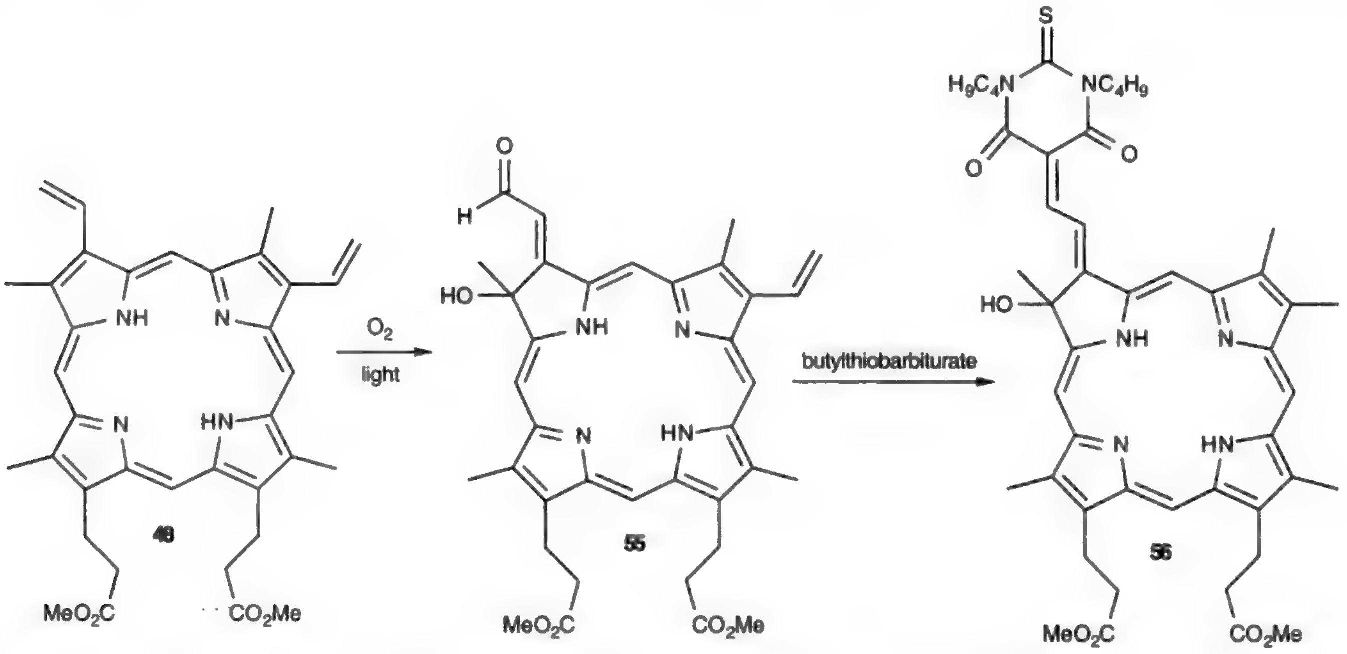
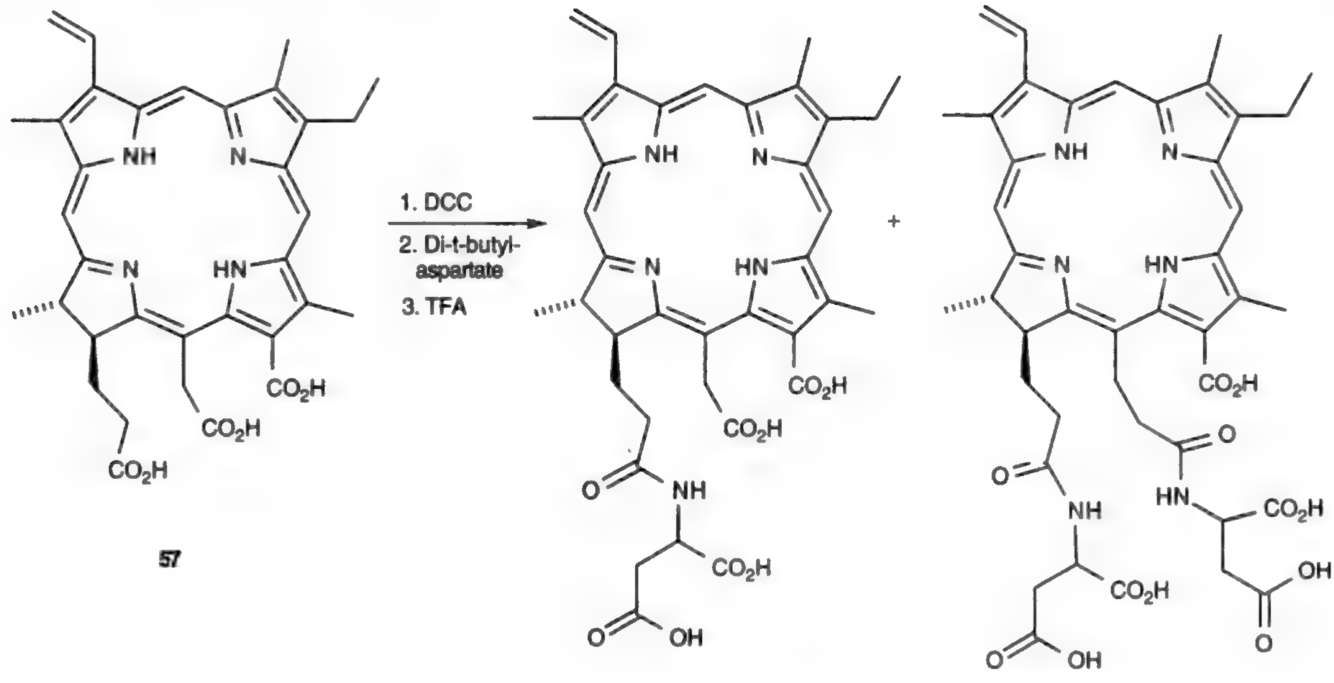


Fig. (15). Singlet oxygen derived from irradiation of PPIX reacts with this sensitizer to give the hydroxy-aldehyde chlorin like species (**55**). This may in turn be derivatized with thiobarbituric acid.



57

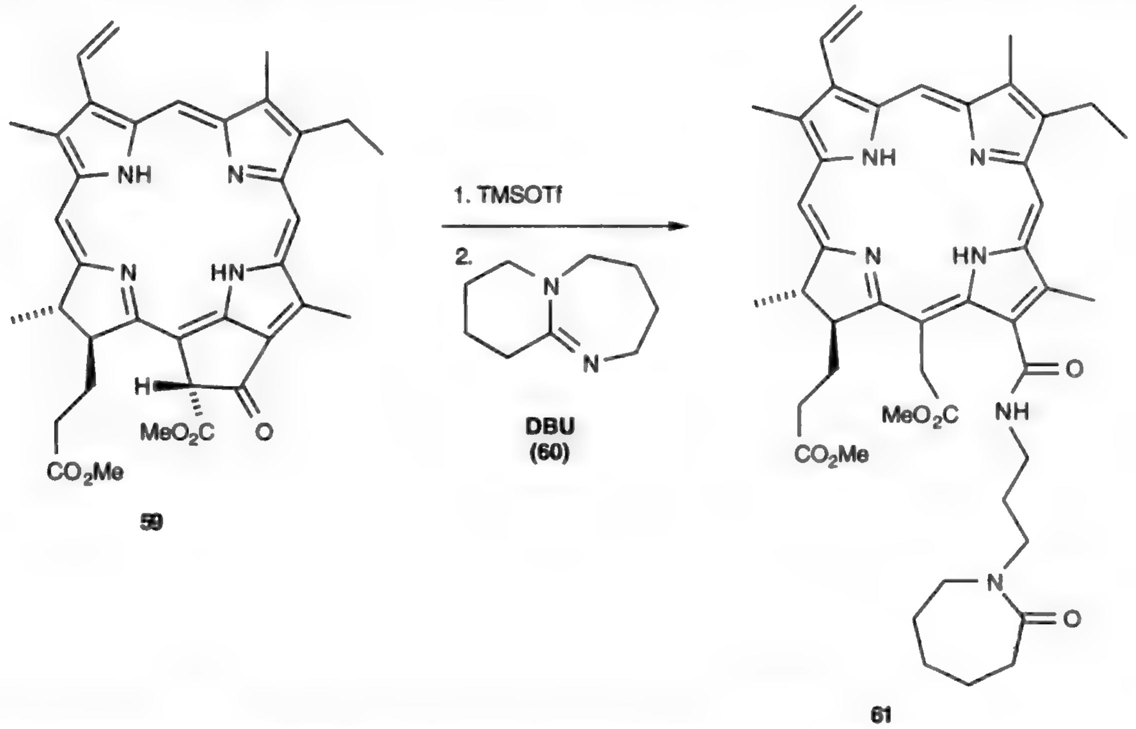
MACE (18)

58

Fig. (16). The di-t-butylester of aspartic acid is conjugated to chlorin e_6 to give either the mono- or di- substituted chlorin. A short treatment with trifluoracetic acid releases the protecting group.

trials today other than PHOTOFRIN®. The main characteristic of this compound is that it has a high affinity for serum albumin to the extent that it can be detected in the circulation for several weeks post injection [126].

A rather unexpected product which leads to a rather more complicated chlorin derivative was generated between methyl pheophorbide a (**59**), and 1,5-diazobicylco[4.3.0.]non-5-ene (DBU, Fig. (**17**)). This is a rather unusual reaction in which DBU was coaxed to act as a nucleophile rather than a base. The chlorin **59** was first treated with DBU (**60**) followed by trimethylsilytriflIate (TMSOTf). This resulted in an electrophillic acitvated species. The addition of DBU to the chlorin results in the opening of its imino cyclic ring

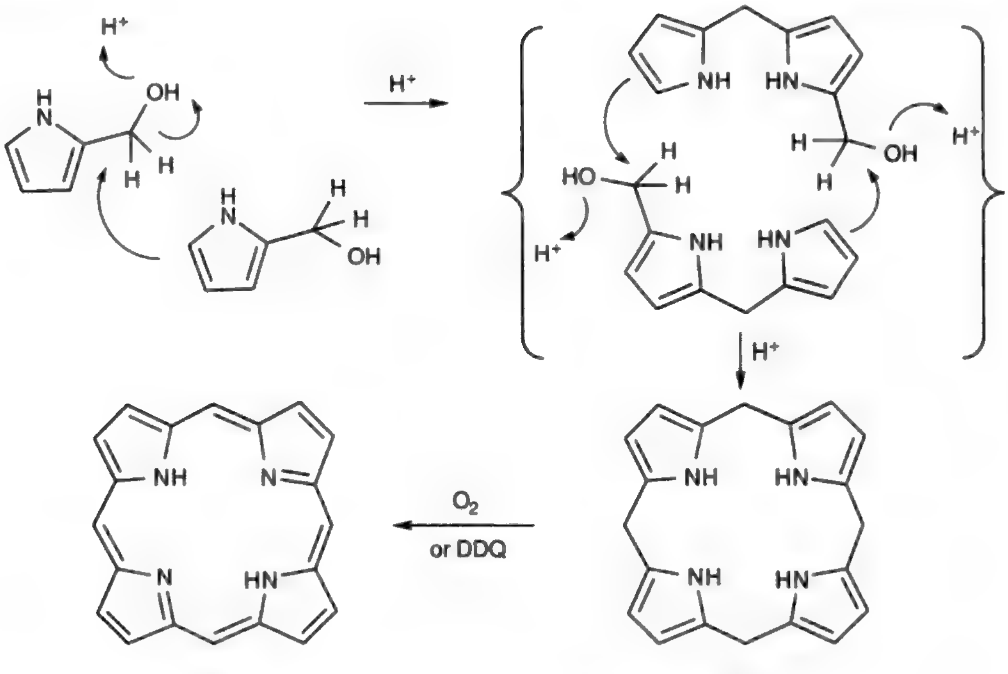


59

DBU (60)

61

Fig. (17). Under certain circumstance DBU may be coaxed to react as a nucleophile with a chlorin.


H+
OH
H
H
H
H
OH
H+
NH
HN
H
HO
H
H
OH
H
H+
H+
NH
HN
H+
NH
N
O2
or DDQ
N
HN
NH
HN
NH
HN
NH
HN

38 **62**

Fig. (18). A porphyrin is formed by the assembly of four hydroxymethylpyrrole units.

along with the addition of its other amine to the 13' keto-position to give **61** [127].

Derivatives of Synthetic Porphyrins

The process to make a porphyrin from four pyrrolic units seems at first glance contra-thermodynamic, Fig. (**18**). However, this is not the case. The reaction proceeds through a stepwise process of first formation of a dipyromethane followed by self condensation, or consequite addition of pyrrole followed by cyclization and then oxidation.

In the typical synthesis of tetraphenylporphyrin (TPP (**64**), Fig. (**19**)), pyrrole is mixed with benzaldehyde and a soluble acid catalyst [128]. The cyclization is then followed by an oxidation to the porphyrin with atmospheric oxygen or by the addition of an oxidant such as DDQ. Until recently the sequence of self-condensation has been thought to occur only through the alpha-carbon of the pyrollic unit. However,

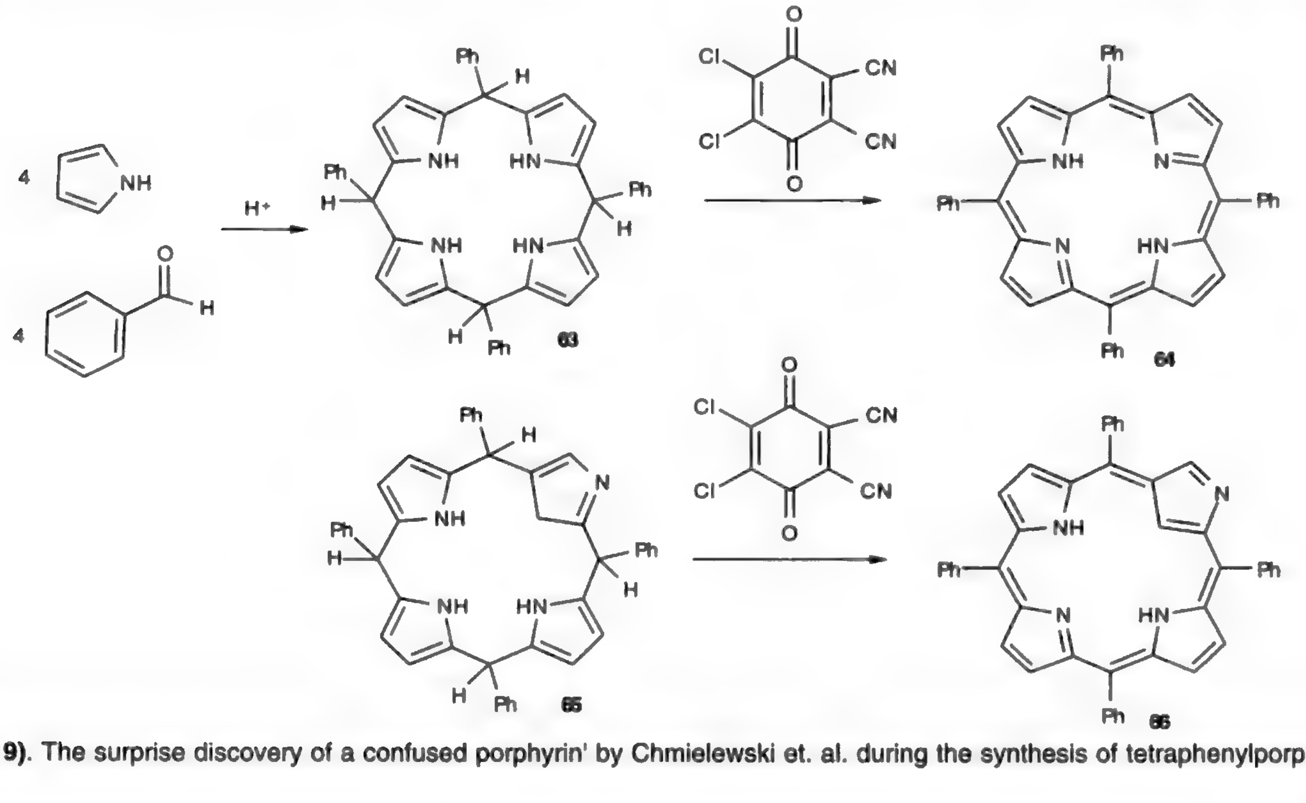

4
NH
4
O
H
H+
Ph
H
NH
HN
Ph
H
Ph
H
NH
HN
H
Ph
63
O
Cl
CN
Cl
CN
O
Ph
NH
N
Ph
Ph
N
HN
Ph
64
Ph
H
N
NH
Ph
H
Ph
H
NH
HN
H
Ph
65
O
Cl
CN
Cl
CN
O
Ph
N
NH
Ph
Ph
N
HN
Ph
66

Fig. (19). The surprise discovery of a confused porphyrin' by Chmielewski et. al. during the synthesis of tetraphenylporphyrin.

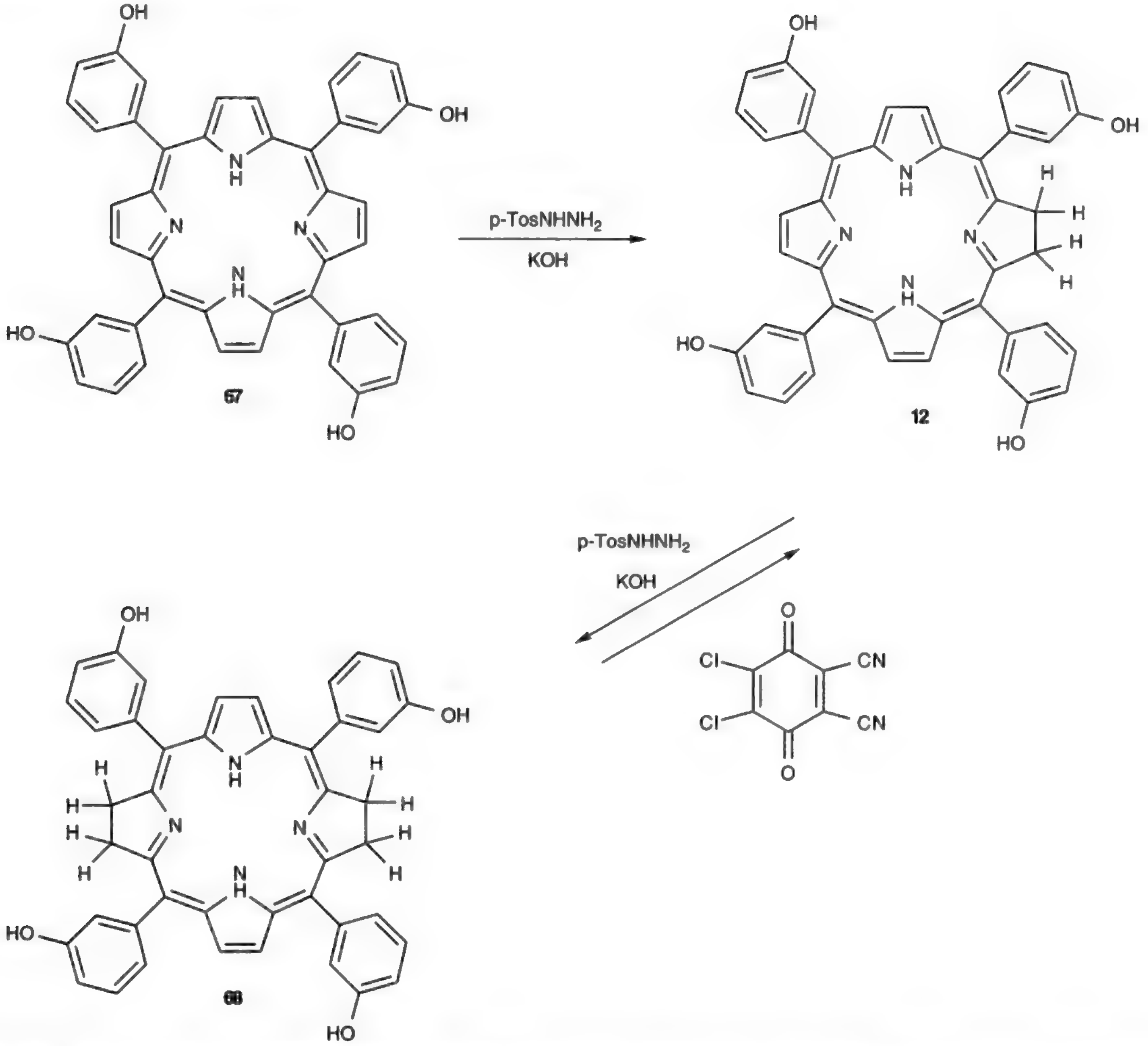


Fig. (20). It is possible to selectively reduce the peripheral double bonds of a porphyrin with diimide to give a chlorin and a bacterochlorin. Oxidation of the bacterochlorin back to the chlorin is possible with the quinone, DDQ.

upon close inspection of a typical TPP synthesis, the formation of the so called 'confused' porphyrin (**66**) in small yields (~5%) can occur [129]. The spectral and physical properties of this isomer of TPP are somewhat different from what one expects of a porphyrin.

In their search for pure compounds as PDT agents Berenbaum and Bonnett began work in 1982 on modified TPP derivatives [130]. Nearly 140 analogs were surveyed in an *in-vivo* tumor response assay. Other criteria such as cost effectiveness and skin sensitivity were utilized to judge the best analog. Based on these results the diimide reduction product of the tetrakis(*m*-hydroxyphenyl)porphyrin (mTHPC) which provided the best combination of properties was the chlorin (**12**) [131], Fig. (**20**). This reduction generates two products, the chlorin and bacterochlorin. However, the bacterochlorin can be readily oxidized back to the chlorin. The possibility or atropisomers derived from restricted rotation around the porphyrin-phenyl ring was unimportant since such rotation is relatively facile at body temperatures [132], as is tautomerization [133].

An interesting variation on the work of Bonnett was performed in our group by Brückner [134], Fig. (**21**). Instead of the reduction of the peripheral double bond of a TPP derivative, an oxidation with osmium tetroxide gave good yields of a diol chlorin species **69** Diaddition was observed to give a mixture of the syn and anti-tetraol **70.** The diol underwent a classic 'pinacol to pinacolone' rearrangement to the known ketone **71** [135]. This work was reminiscent of the studies performed by Chang's group on octaethylpoprhyrin [136].

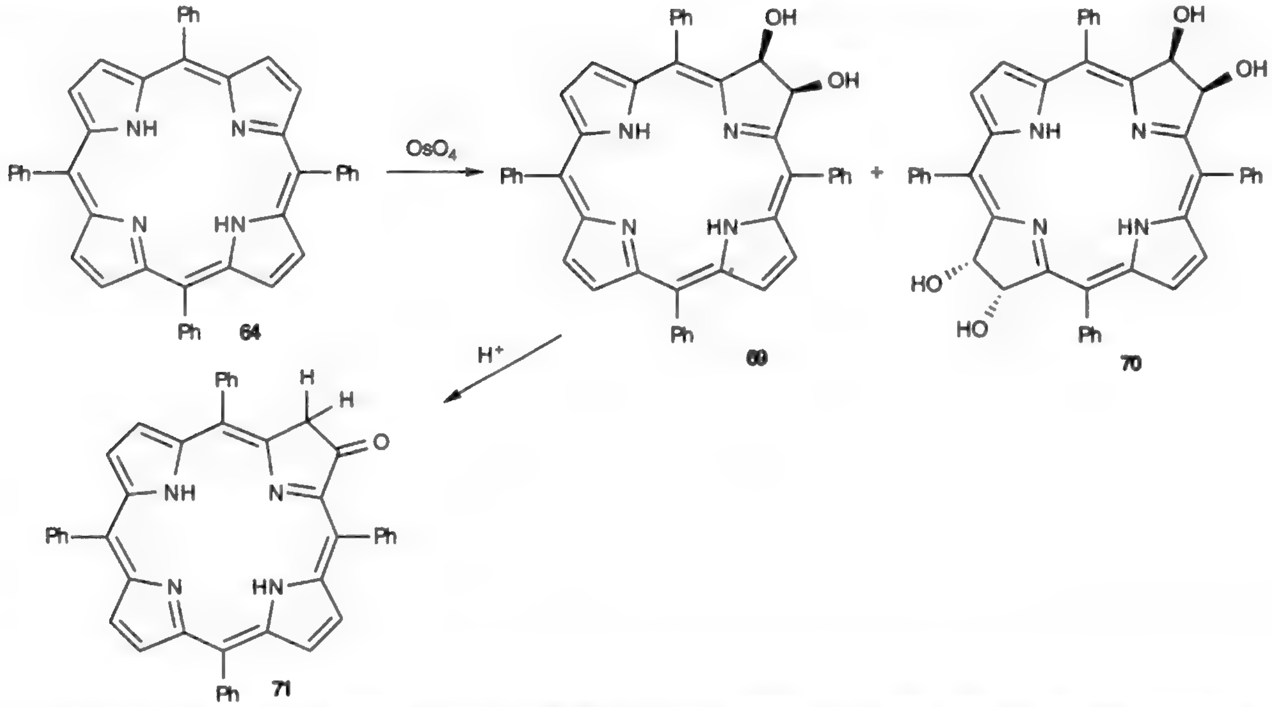


Fig. (21). Instead of reduction an oxidation can be performed on the peripheral double bonds to give the dihydroxychlorin or the tetrahydroxybacterochlorin. Treatment of these with acid initiates a rearrangement to the ketone.

Other alkylporphyrins have been dervivatized and have been found to be effective PDT agents. Etioporphyrin (**72**) was treated with **73** under Vilsmeyer-Haak conditions to give exclusively the isomeric derivative **74**, Fig. (**22**). Under strong acidic conditions the compound rearranges to the purpurin **75**. This was metallated to give the in dichloride purpurin [137]. Several interesting chemical questions

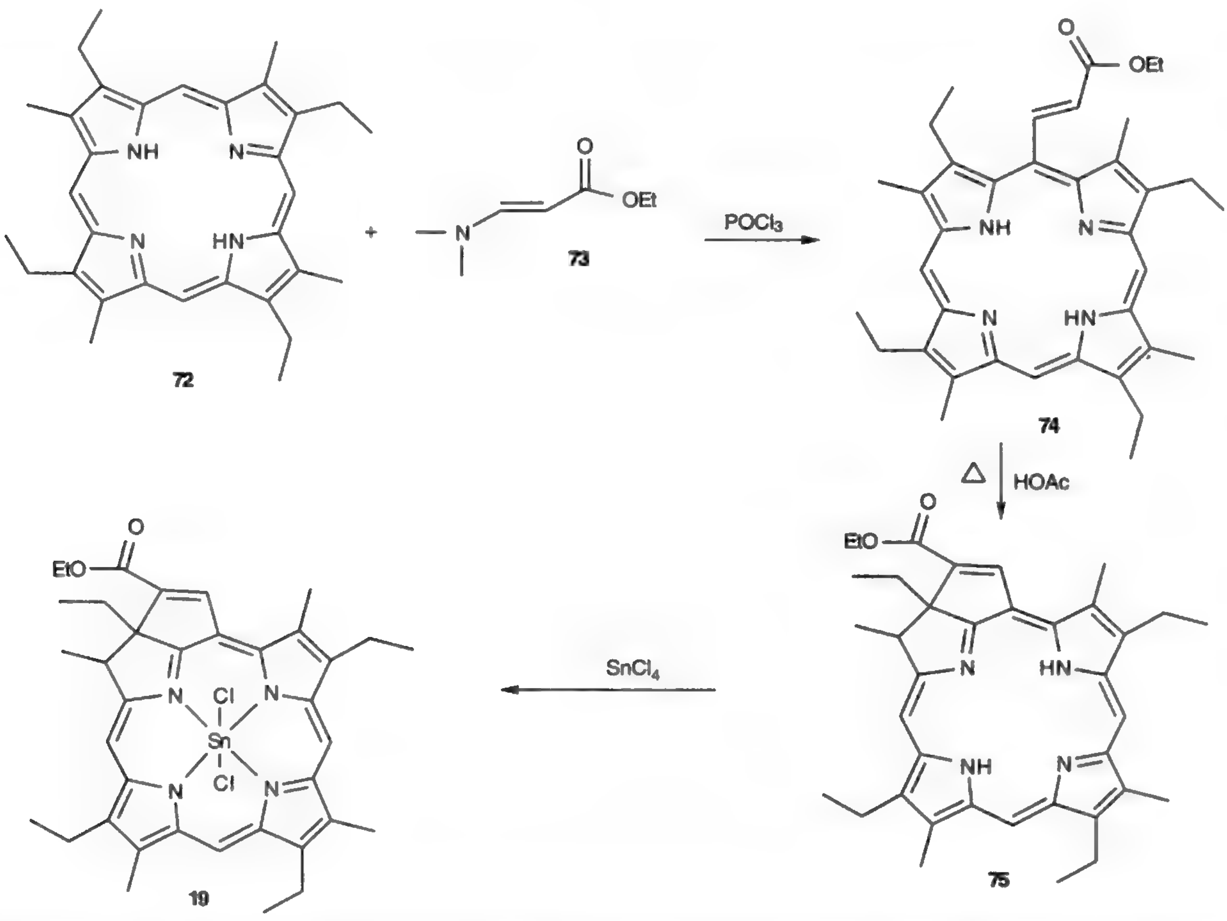


Fig. (22). Porphyrins can be undergo a vinyligous Vilsmeyer reaction at the methyne position. Rearrangement to the purpurin is catalized by acid. Metallation with $SnCl_4$ proceeds readily.

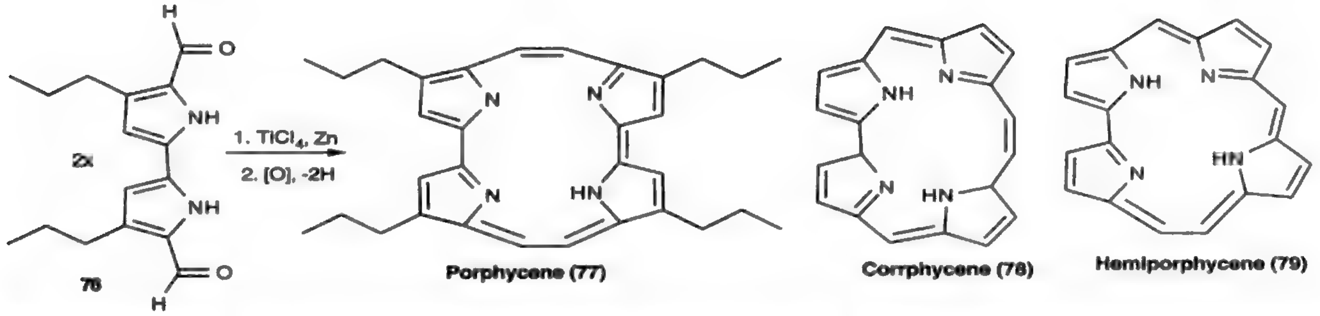


Fig. (23). An isomer of a porphyrin is available through the McMurry coupling. Other isomeric analogues of porphyrins have also been produced.

are raised by this compound. These include selectivity of the cyclization to give only the one isomer towards the ethylgroup, along with the stability and fate of the axial coordinated chlorides on the body.

Unusual Ring Systems

A rather unique application of the McMurry coupling of two bipyrrole dialdehydes allowed Vogel and his colleagues to create a ring system isomeric with a porphyrin [138],.Fig. (**23**). These porphycenes (**77**) gave a Q-band which can be shifted to the red by about 35 nm depending on the derivative. An array of metallated versions of this compound have been prepared along with analogs which exhibit better solubility characteristics. The structural isomer to TPP where phenyl groups lie between the pyrroles has also been produced [139]. Glaxo-Wellcome in collaboration with Cytopharm have judged these systems adequate PDT agents for at least application on the skin. Other 'porphyrin like' isomers which have been synthesized are hemipophyrcene [140] (**79**) and corrphycene [141] (**78**).

The utilization of phthalocyanines as dyes harkens back to the 1930's. These materials were found to possess incredible stability in the presence of light and oxygen. This is most likely due to their highly aggregated state even in solution. The major interest in these compounds today is their application as inks in inkjets, in photo-offset applications as dye pigments and for their electical properties. As with other extended aromatic species, in their monomeric state they have the ability to generate singlet oxygen. The treatment of a bis-phthalonitrile with ammonia results in a heterocyle which has a uniquely distinctly blue color, Fig. (**24**). Often the cyclization is run in the presence of

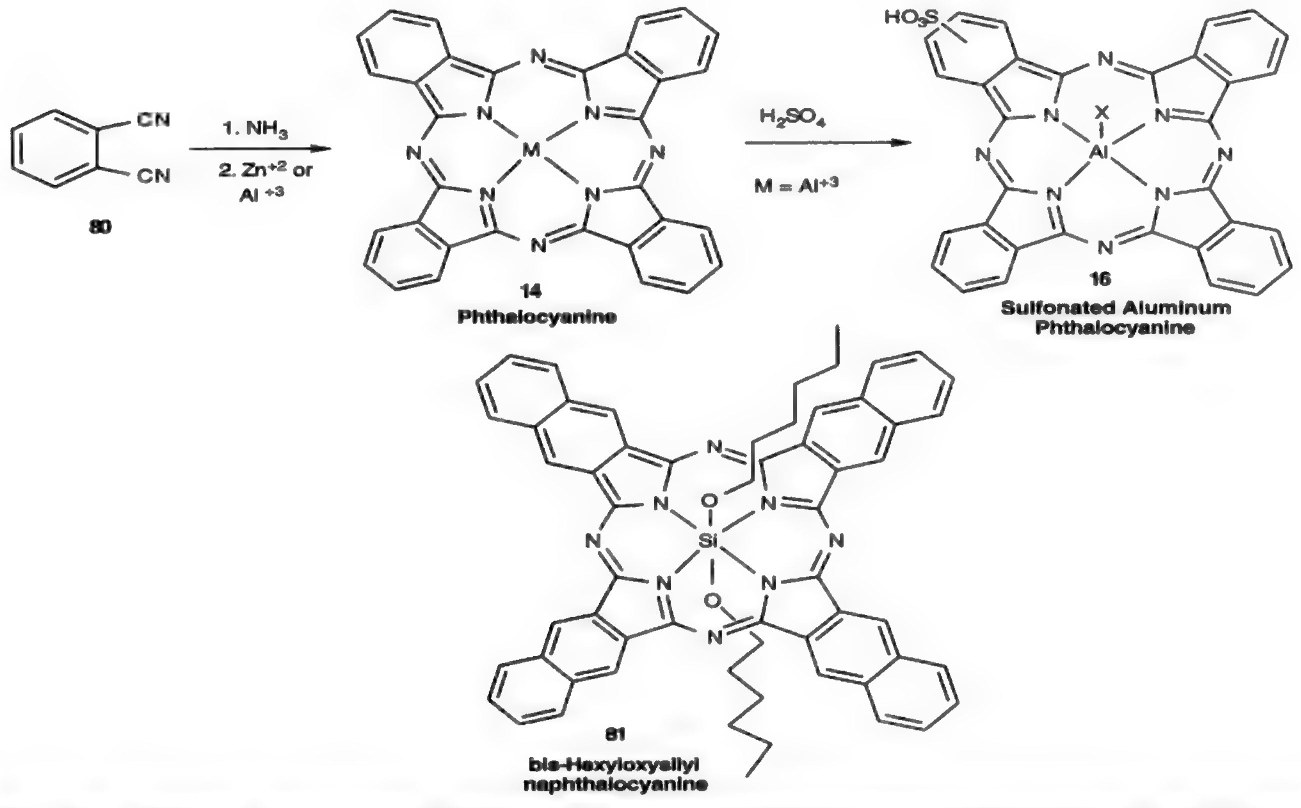

CN
CN
80
1. NH$_3$
2. Zn^{+2} or Al^{+3}
N
M
14
Phthalocyanine
H$_2$SO$_4$
M = Al^{+3}
HO$_3$S
X
Al
16
Sulfonated Aluminum Phthalocyanine
O
Si
81
bis-Hexyloxyallyl naphthalocyanine

Fig. (24). In the presence of ammonia the bis nitrile cyclizes to the phthalocyanine. Naphthalocyanines are the result of an ammonia reaction with bis-cyanonaphthalene.

metals, thus adding greatly to the possible number of analogs. Metal-like species such as hexavalent Si add the unique ability to functionalize these heterocycles through axial ligand coordination. Brasseur et al have explored the utilization of bis-(dimethylhexylsiloxy)-naphthalocyanine (**81**) in the field of PDT [142]. The simplist compound undergoing clinical trials today is Zn-phthalocyanine. However, it provides a unique problem in formulation since it exists as a aggregate except in extremely strong solvents such as pyridine. The compound has for a number of years been known to be an effective phototoxin. It was the Herculean tasks to formulate it in a liposome which directs the drug to LDL's which allowed Ciba-Geigy to obtain a patent position for this species [143].

Much like the phthalocyanine, the porphacyanines **84**, Fig (**25**), which were developed in our group, contain heteroatoms in the periphery of the extended aromatic system [144,45]. The formation of the ring

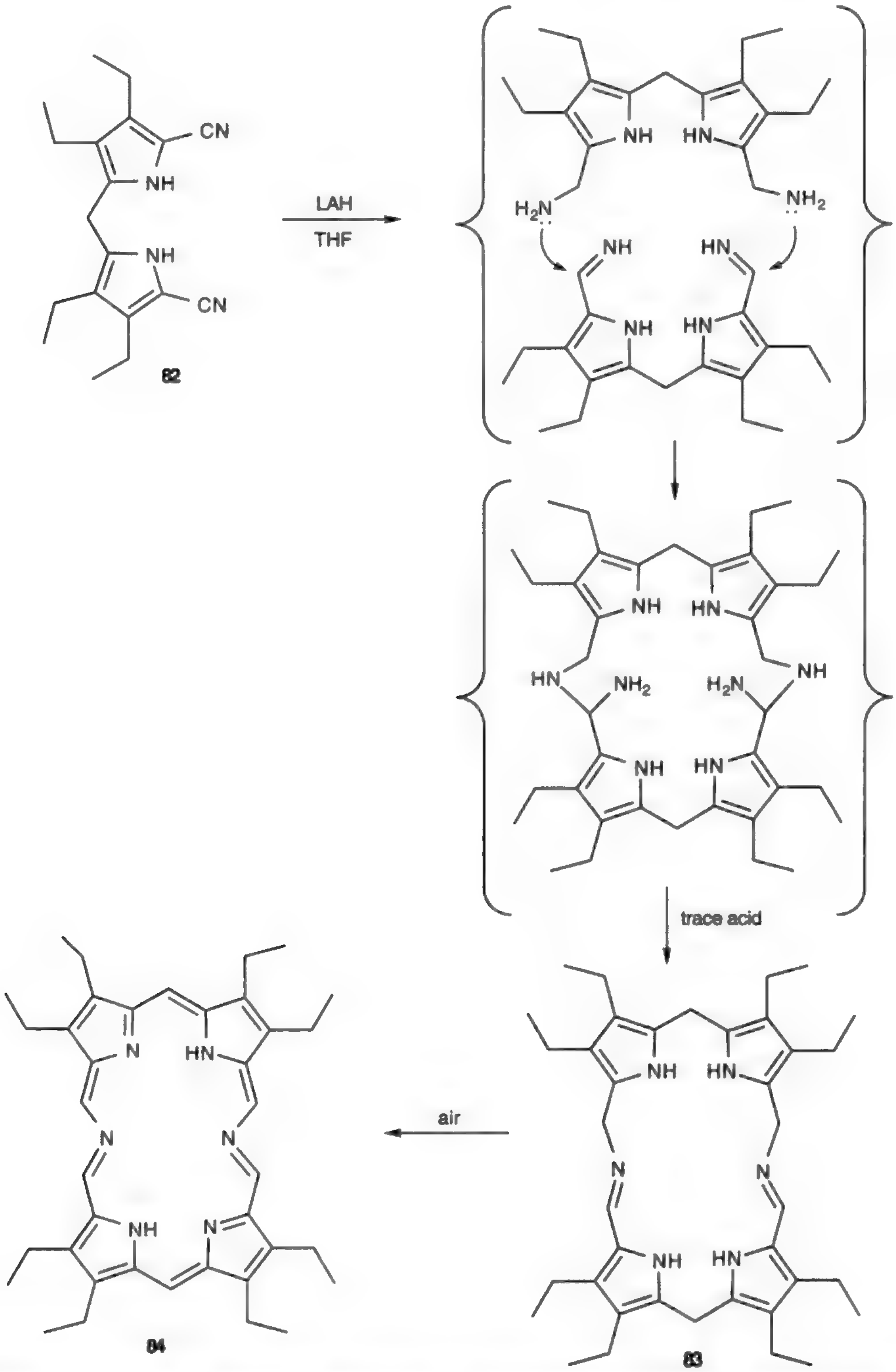


Fig. (25). Upon reduction with lithium aluminum hydride the bis-nitrile is reduced to a diamine which, in the presence of oxygen, oxidizes to the imine, whereupon cyclization occurs.

occurred in good yield via a rather surprising series of events which are still being investigated. This large macrocycle was produced upon reduction of the bis-nitrile **82** and subsequent oxidation with either air or treatment with DDQ. Two intermediates which were thought to result in macrocycle formation are the fully reduced amine and the partially reduced imine. The amine condenses onto the imine to begin the macrocycle formation. Several analogs have been produced which are presently under investigation for their application in PDT.

A rather unique heterocycle was formed by Sessler's group [146] by the cyclization between the diamimo compound **86** and the tripyromethane species **85**, Fig. (**26**). A variation on this basic theme is also shown in structure **89**. The expanded aromatic unit has been found to be readily metallated by lanthanides. These metals are too large to enter into a standard porphyrin and therefore sit on the edge of a porphyrin core. This is not the case for expanded porphyrins. A variety of these metallated species are being investigated for both radioactive and magnetic resonance imaging as well as for PDT.

This ability to metallate a porphyrin with most metals or metalloids in the periodic table allows another level of derivatization not normally seen with standard drugs. These metals include but are not limited to Ge [147], ^{55}Fe [148], ^{67}Ga [149]. Depending on the metal a variety of properties can be explored. These include the addition of axial ligands onto the metal, the distortion of the plane of the porphyrin, and changes in the electronic nature of the spectrum. One can also

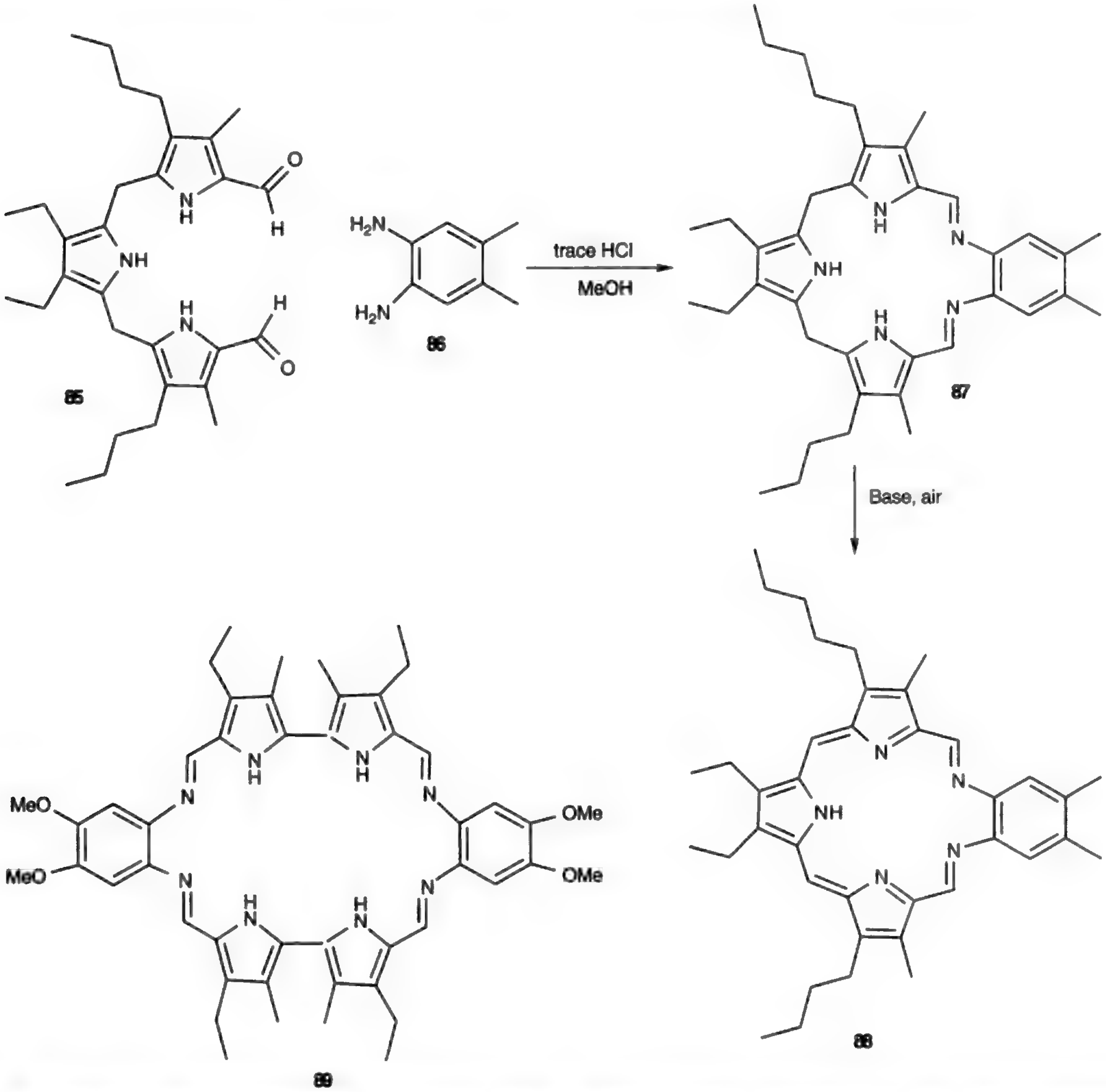


Fig. (26). Texaphyrin and other similar macrocylcles are the result of reactions between an amine and an aldehyde to generate an imine.

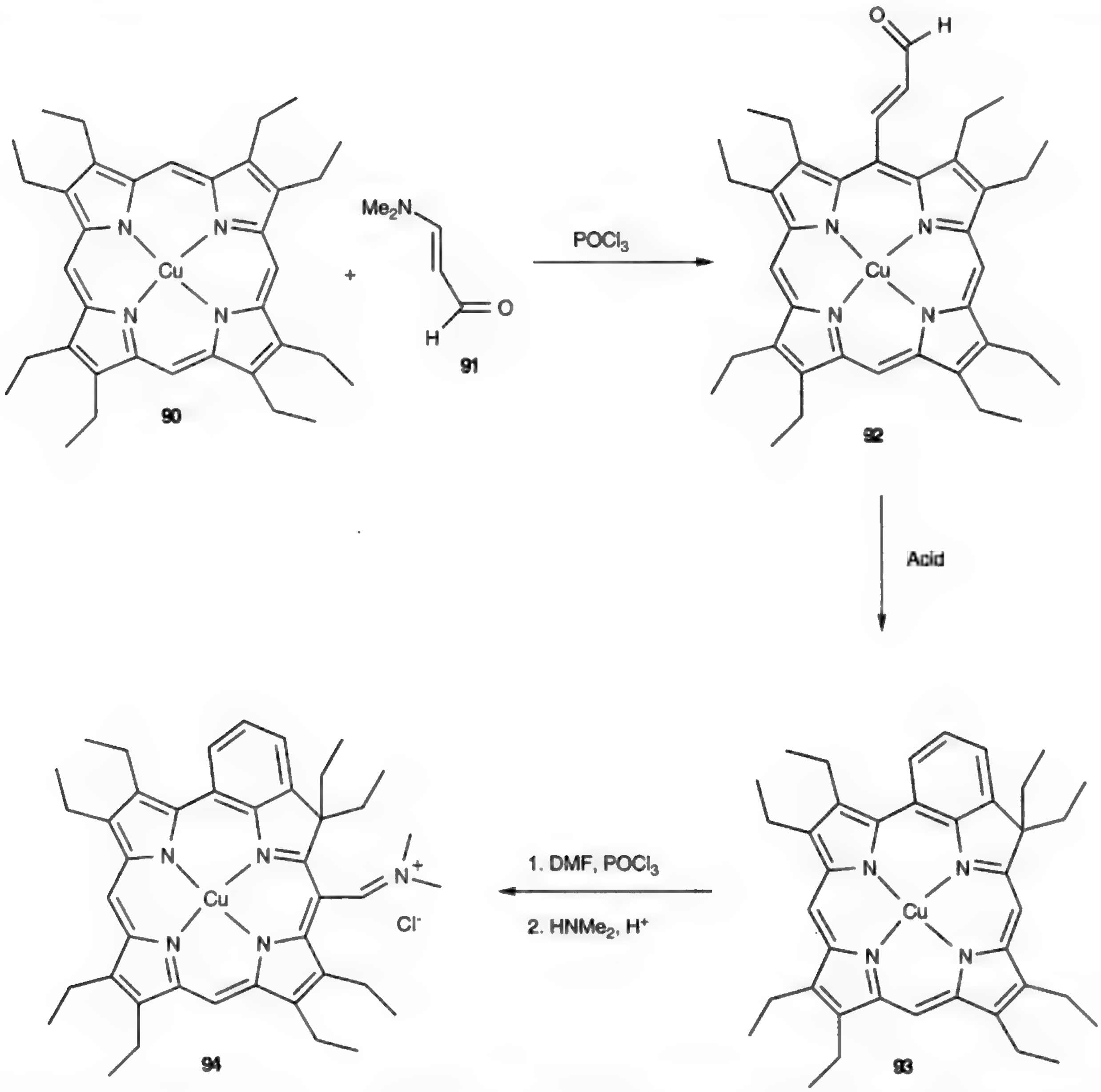


Fig. (27). The copper complex can be modified through a Vilsmeyer reaction followed by formation of the imminium salt.

utilize this ability to change a singlet oxygen sensitizer to one which is phototoxic via another process such as seen with compounds made by Morgan's group [150],Fig. (**27**). The copper in the porphyrin is known to quench the triplet state. However, upon irradiation the material can still cause localized toxicity via electron transfer or crosslinking mechanisms.

Clinical Introduction

The clinical applications of PDT seem to be at least as vast as the number of possible PDT drugs. These not only include many types of cancer but because of the immunomodulatory effect of the drugs, along with the manipulation of localized vasculature, they include at least the following [151-153].

1. Cancer
    a. Esophag [154], Other head and neck, Lung, Bladder
    b. Skin- Basal cell, Squamous cell, Kaposi's [155], Melanoma, Bowen's
    c. Others- Colon, Prostate, Mesothelioma, Glioma, Melanoma of Eye, Mesothelium of the chest
2. Eye- Age related Macular Degeneration, Adjunctive to Glaucoma Surgery
3. Cardiac- Restenosis [156,157]
4. Skin- Psoriasis, Acne, Hair Removal, Actinic Keratoses, Port Wine Stains

5. Infectious Agents- HIV [158], Human Papilloma [159],"Anti-Bacterial", Malaria

6. Misc. - Arthritis [160], "Auto-Immune" diseases, Bone Marrow Purging, Endometrium, Gynecological

At one time or another PHOTOFRIN® or its precursor HpD has been investigated in some manner in regards to most of the above indications. Realistically, the clinical applications which seem to hold the most promise for development and financial reward are in the areas of head, neck and lung cancers, along with macular degeneration of the eye (AMD), all skin cancers and psoriasis. These have all been investigated extensively in clinical settings. There is one common thread which seems to hold these rather disparate areas together. The development of excessive or unusual vasculature which when destroyed can moderate the disease. The selectivity of PDT in regards to these areas is dependent upon the uptake and then specific irradiation of the region of disease. The architecture of most of these diseases are rather flat and thin, except for lung cancer tumors which range to greater than 1 cm in diameter. Therefore, the application of light to the area does not require excessively deep penetration to initiate a therapeutic response. Recently the compounds undergoing clinical trials for some of these diseases are irradiated with blue light to restrict damage to supporting tissues.

The special considerations needed to evaluate a clinical drug for PDT are the period of residual skin photosensitivity, the time after introduction of the drug at which light irradiation begins, the time of irradiation, light dose, wavelength of irradiation and the method of drug introduction and of course therapeutic response. These will determine whether a drug is appropriate for its proposed purpose.

PHOTOFRIN® (QLT PhotoTherapeutics)

PHOTOFRIN® is a solid red powder which is normally brought into solution in dextrose to a concentration of 2 mg/mL. Typical dosages are 2.0 mg/Kg with treatment beginning 40-50 hours after injection. Irradiation wavelength is at 630 nm with either an argon pumped dye laser or a frequency doubled YAG pumped dye laser and a total dosage of 200-300 joules. Skin photosensitivity lasts about 30 days.

The number of patients treated over the past years with this drug easily exceeds 5,000. The number of clinical publications associated with these trials are vast. Results summarized by Marcus [161] give an indication of the drug's success in the clinic. In studies covering several hundred patients, the complete response (CR) levels for obstructive lesions were 50% moving to 76% for mucosal lesions. Including the partial responses (PR) one observes a 75 and 78% response level, respectively, for these lesions. There was some indication that glucocordicoids given to relieve swelling caused a poorer response which might be an indication that the immuno-modualtion aspects of PDT were being affected. The clinical status of PHOTOFRIN® is shown in Table 5.

Table 5: Clinical Status of Photofrin

Indication	Status	Date
Superficial Bladder	Approved/Canada	1995
Obstr. Lung and Esophageal	Approved/Netherlands	1994
Early Gastric, lung, Esophageal	Approved/Japan	1995
Esophageal	Approved/U.S./Canada	1995
Lung and Esophageal	Europe Pending	1995/96
Barrett's/oral	Phase I	-

Photosan III (Seehof Laboratories)

A hematoporphyrin derivative with similar structural properties to those of PHOTOFRIN®. This compound has been studied almost exclusively in Europe in nearly 700 patients [152].

Benzoporphyrin Derivative (QLT PhotoTherapeutiecs)

BPDMA (verteporfin) has progressed to phase II clinical trials which have included treatment of non-melanoma skin cancer [162] and psoriasis [162]. Additionally, in association with CIBA Vision Opthalmics Inc. a multicentered trial exploring its use in age related macular degeneration (AMD) is underway [163]. The typical dosage of this green compound in its liposome formulation for cancer or psoriasis is 0.25 mg/Kg and somewhat less for AMD. Irradiation for tumors at 690 nm begins at 3 hours after injection while for AMD irradiation begins almost immediately. The intensity of irradiation is about 150 joules while much less for AMD where solid state lasers are used in the clinic. Total time of photosensitiviy rarely exceeds 3 days for the dose routinely used for cancer treatments.

A single treatment regime with BPDMA and red light utilizing a bank of light emitting photo-diodes (LED's, emitting at ~690 nm) for psoriasis showed a definite improvement in the plaque's structure (flaking, thickness, redness) for periods up to 90 days [162]. It is felt that this treatment might require a multiple dosage routine. However, it is expected the number of these would be substantially less than the typically 20-30 treatments required for PUVA therapy.

Treating non-melanoma skin cancers with drug dosages from 0.15 mg/Kg to 0.50 mg/Kg and light dosages ranging from 50 J/cm^2 to 150 J/cm^2 gave a complete response rate of 87% with an additional 4% partial response [162]. Less variation in the dosage/response profile is observed by basing the dose on mg/M^2. Light systems are also being changed to LED's rather than the typical laser based therapy. This is less costly and allows more freedom of treatment in a clinical setting.

By far the most exciting area of therapy with regards to this drug is associated with the treatment of AMD [163]. This disease is a result of excessive neo-vascularization of the retina and affects a great number of the aging population. It accounts for a majority of this population's blindness. The trials to date have treated in excess of 100 patients giving encouraging results. The extent of the vascular shutdown is presently being assessed.

ALA (Dusa Pharmaceuticals and Deprenyl Research)

This pro-drug is formulated in aqueous solution for oral activity while several topical formulations have been developed. The quantity of the drug dose ranges to 200 mg/Kg for oral dosages while topical formulations can exceed 20%. As a pro-drug several biochemical processes which typically takes several hours must take place before the photoactivatable drug, protoporphyrin IX, is formed. The PPIX is shortlived in tissues because of the bodies ability to eliminate or modify it. No major toxicity has been associated with this drug. However, since ALA accumulates in nerve cells and then is converted to active drug, treatment can often be quite painful. Irradiation occurs at ~630 nm a wavelength associated with moderate penetration. The number of trials on this drug exceeds that of any other photosensitizers besides PHOTOFRIN®. This is one of the few photosensitizing drugs which has absolutely no vascular component to its therapeutic response, probably because it is produced inside the cell.

No summary of the at least 60 clinical trials is available at this time. ALA has been applied to basal cell carcinoma [164], psoriasis [165] and actinic keratoses [166]. Diagnosis via the production of protoporphyrin has been applied to Barrett's esophagus, colorectal, bladder and brain cancers.

Tetrahydroxytetraphenylchlorin (Scotia Pharmaceuticals)

This phenolic chlorin has a wavelength of excitation at 652 nm and can be activiated with a copper vapor laser. Drug dosage was typically 0.15-0.30 mg with a concurrent light dosage of 20 joules at a period of 96 hours after injection. Skin sensitivity ranged to a period of up to 3 weeks. The pharmacokinetic profile seems to to be affected by aggregation of the drug since there seemed to be bimodal clearance [167] and even large variation among the same individuals. This is perhaps the result of the formulation which consists of polyethylene glycol, water and ethanol.

A clinical trial treating esophagus and bronchi which contained 12 patients showed that after several months 7 had no evidence of tumor and 2 had a recurrence [168]. Further work by Dilkes and DeJude [169] treating 17 patients with widely differing types of cancer showed good to moderate responses. There is noted a danger of perforation of the esophagus when irradiating at 652, so that the wavelength of irradiation has been changed to 514 nm for this purpose [170]

Tin Etiopurpurin (PDT Inc. and Pharmacia & Upjohn)

Typically formulated in an emulsifying agent, this compound has very little published literature about its progress through clinical trials. It has been investigated mostly for Kaposi's sarcoma [171] The major absorption peak for irradiation is at 660 nm. Initial characteristics show that maximum accumulation occurs at 24 hours. Photosensitivity may last beyond one month.

Mono-Aspartylchlorin e6 (Nippon Petrochemical and Meiji Seika Kaisha)

There is very little information about clinical trials. Some trials have occurred at the U. of California at Davis. There have been reports of further trials in Japan. From some animal data, one can be subjective about its properties. The formulation is aqueous at a concentration of 1.0 mg/mL reconstituted at site. The primary wavelength of irradiation is at 664 nm and the solution is very photo-labile. The drug is easily cleared from the body and therefore, photosensitivity rarely lasts beyond a few days. However, it is believed that at least some of the drug stays in the serum albumin for long periods. There seems to be no vasculature affect for this tetracid probably as a result of its high change density.

Porphycenes (Cytopharm and Glaxo-Wellcome)

The tetra ether **20** is presently being developed as a topical agent for the treatment of psoriasis. This compound also has been formulated in a liposome. It has begun clinical trials as of now mostly in Europe [172].

Photosens (Russia)

This is a mixture of sulfonated aluminum phthalocyanines which is irradiated at about 670 nm with a pulsed frequency doubled Nd:yttrium aluminate laser [173]. This is a drug which would seem to have no patent position since sulfonated phthalocyanines have been investigated for years. The likelihood that a drug that is a large mixture gaining approval is not great outside of Russia.

A variety of tumors were treated in a series of trials on 45 patients. Drug dosages were from 0.5 to 2.0 mg with light dosages ranging to 175 J. Irradiation times after injection were 24-72 hours. The CR rate for 120 tumors was 56%. All the additional tumors showed some response to the treatment.

Zn Phthalocyanine (QLT PhotoTherapeutics and CIBA Vision Opthalmics)

One of the metallated species seriously being considered for PDT is zinc phthalocyanine **14** which is perhaps the simplest of all compounds discussed herein. Zinc phthalocyanine is insoluble in just about all solvents except pyridine. However, workers at Ciba-Geigy have managed to formulate it into liposomes. QLT PhotoTherapeutics in co-operation with CIBA Vision are investigating it in the field of AMD.

Texaphyrins (Pharmacyclics)

The last serious photosensitizer in clinical trials is a texaphyrin (**15**). It is known that at least one Gd metal-derivative is being utilized as a radiosensitizing agent in a Phase I/II clinical trials while a Lu metal-derivative is under investigation in a Phase I trial for restenosis.

Clinical Summary:

Comparison of the clinical efficacy of the different photosensitizers on gastrointestenal tumors has only just begun. In a recent study by Milkvy [174] the response to the three compounds, ALA, PHOTOFRIN®, and mTHPC was assayed. In the case of ALA only superficial lesions (1.8 mm deep) were treated, while with PHOTOFRIN® and mTHPC larger tumors and polyps were successfully treated.

Conclusions

Photodynamic therapy has moved from a novel therapeutic idea to a viable treatment modality with the approval of PHOTOFRIN® in several jurisdictions throughout the world.

Acknowledgement

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Novel Effects of Heme and Heme-related Compounds in Biological Systems†

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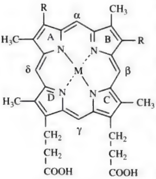
Abstract: Heme and heme-related compounds such as porphyrins have a variety of biological and clinical activities. This review highlights some of the novel effects of these compounds in medicinal chemistry. Heme which has no photoreactive properties, can serve as the prosthetic group for various hemoproteins, or as a substrate for microsomal heme oxygenase (HO). In contrast, porphyrins have photodynamic activities but usually do not serve as substrates in biological systems. Thus, heme and heme analogues are often used to influence enzyme activities in tissues, while porphyrins by taking advantage of their photoreactive properties are used as drugs in medicine. There are four major applications of these compounds, namely the use of (i) heme in the treatment of porphyrias and hematological disorders, (ii) synthetic metalloporphyrins in the treatment of hyperbilirubinemia, (iii) metal-free porphyrins in photodynamic therapy of malignant tumors, and (iv) porphyrin derivatives as contrast reagents in radiography. Solubility, stability, pharmacokinetics, pharmacodynamics and analytical techniques are all important considerations in the medicinal chemistry of these compounds. In man and animals *in vivo* , the rate of synthesis and catabolism of heme, as well as disorders of heme synthesis are important in understanding pathophysiological effects of these compounds.

Introduction

Heme is ferrous protoporphyrin IX and is the prosthetic group of hemoproteins. The structure of heme and its important analogues is shown in Fig. **1**. Heme is synthesized by enzymes in the heme biosynthetic pathway, which are localized in part in the mitochondria and in part in the cytosol.

Porphyrins are molecules of ancient derivation. For example, corrin, a porphyrin derivative used in the backbone of vitamin B_{12}, probably existed in early



PP: $M = 2H$, $R = -CH = CH_2$
Heme: $M = Fe$, $R = -CH = CH_2$

MP: $M = 2H$, $R = -CH_2 - CH_3$
Mesoheme: $M = Fe$, $R = -CH_2 - CH_3$

Sn-PP: $M = Sn$, $R = -CH = CH_2$
Sn-MP: $M = Sn$, $R = -CH_2 = CH_3$

HP: $M = 2H$ $R = -\underset{\overset{|}{OH}}{CH} - CH$

Fig. (1). Structure of heme and important analogues.

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†This article is dedicated to the fond memory of the late Rosalia B. Frydman, Professor Biochemistry, Facultad de Farmacia y bioquimica, Universidad de Buenos Aires, Argentina, who has made truly seminal contributions in the research of heme biology, pyrrole chemistry and/or oxygenases.

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Table I. Applications of Heme and Heme-Related Compounds in Medicine

Compound	Type of application	Reference
Hematin (inc. heme arginate)	Acute intermittent porphyria	[26,38,154]
	Congenital erythropoietic porphyria	[39)
	Hereditary coproporphyria	[50)
	Variegate porphyria	[50,154]
	Lead poisoning	[155)
	Tyrosinemia	[156]
	Sideroblastic anemia	[39]
	Myelodysplastic syndrome	[65]
Sn-protoporphyrin/Sn-mesoporphyrin	Neonatal jaundice	[70]
	Gilbert's syndrome	[147]
	Crigler-Najjar syndrome	[72]
	Primary biliary cirrhosis	[147]
	Psoriasis	[73]
Photofrin II, hematoporphyrin, ALA	Bladder tumor, esophageal cancer, lung cancer, skin cancer, ocular tumor, brain tumor	[74,75,157,158]
Boronated metalloporphyrin	Boron neutron capture therapy (melanoma,angiosarcoma)	[97)
Mn-tetraphenylporphine sulfonate	Contrast agent and/or enhancer in MRI	[101,103,104,159]

bacteria 3.8 x 10^9 years ago [1]. It is, however, only recently that they have found some medical and medicinal use, and the clinical potential of these compounds has not been fully investigated. The therapeutic applications known to date of heme and heme-related compounds are summarized in Table I.

Structural Aspects of Useful Heme and Heme-Related Compounds

The structures of therapeutically important porphyrins can be classified as follows: those which contain iron at the center of the porphyrin macrocycle, i.e., heme and its analogues, and those which do not contain iron, i.e., metal-free porphyrins and non-heme (non-iron) metalloporphyrins. Among heme analogues, those which are effective in influencing enzyme activity such as HO carry two propionate residues at C6 and 7 (Fig. **2**), while other isomeric forms of iron protoporphyrin are not substrates or inhibitors of HO [2]. Thus, a hemin with only one propionic acid residue at the two vicinal propionic acid residues in rings C and D is not a substrate of HO [2]. The IX isomer structure is also essential for non-iron metallopor-phyrins which are used as HO inhibitors. In contrast to the IX isomeric configuration, substitution of peripheral positions, particularly in the rings A and B, does not significantly change the biological activity of these compounds. For example, replacement of the vinyls in rings A and B by methyl groups results in hemins which are excellent substrates of HO [2].

Biosynthesis of Heme and Porphyrins

The enzymatic steps and intermediates in the heme biosynthetic pathway are illustrated in Fig. **2**. In animal cells, the first step, and the last three steps occur in mitochondria; the intermediate steps take place in the cytosol. The two major organs which are active in heme synthesis are the liver and the erythroid bone marrow, and inherited enzymatic defects in the porphyrias are mainly expressed in these tissues.

δ-Aminolevulinate Synthase (ALAS)

ALAS is the first enzyme of the heme biosynthetic pathway and catalyzes the condensation of glycine and succinyl CoA to form δ-aminolevulinic acid (ALA) (Fig. **2**, step 1). The enzyme is localized in the inner membrane of mitochondria and requires pyridoxal 5'-phosphate as a cofactor (3). ALAS activity is very low, and is rate-limiting for heme formation [4]. Hepatic (or non-specific) and erythroid ALASs are isozymes which are encoded by two distinct nuclear genes [5,6], i.e., ALAS-N and ALAS-E genes, respectively. The two

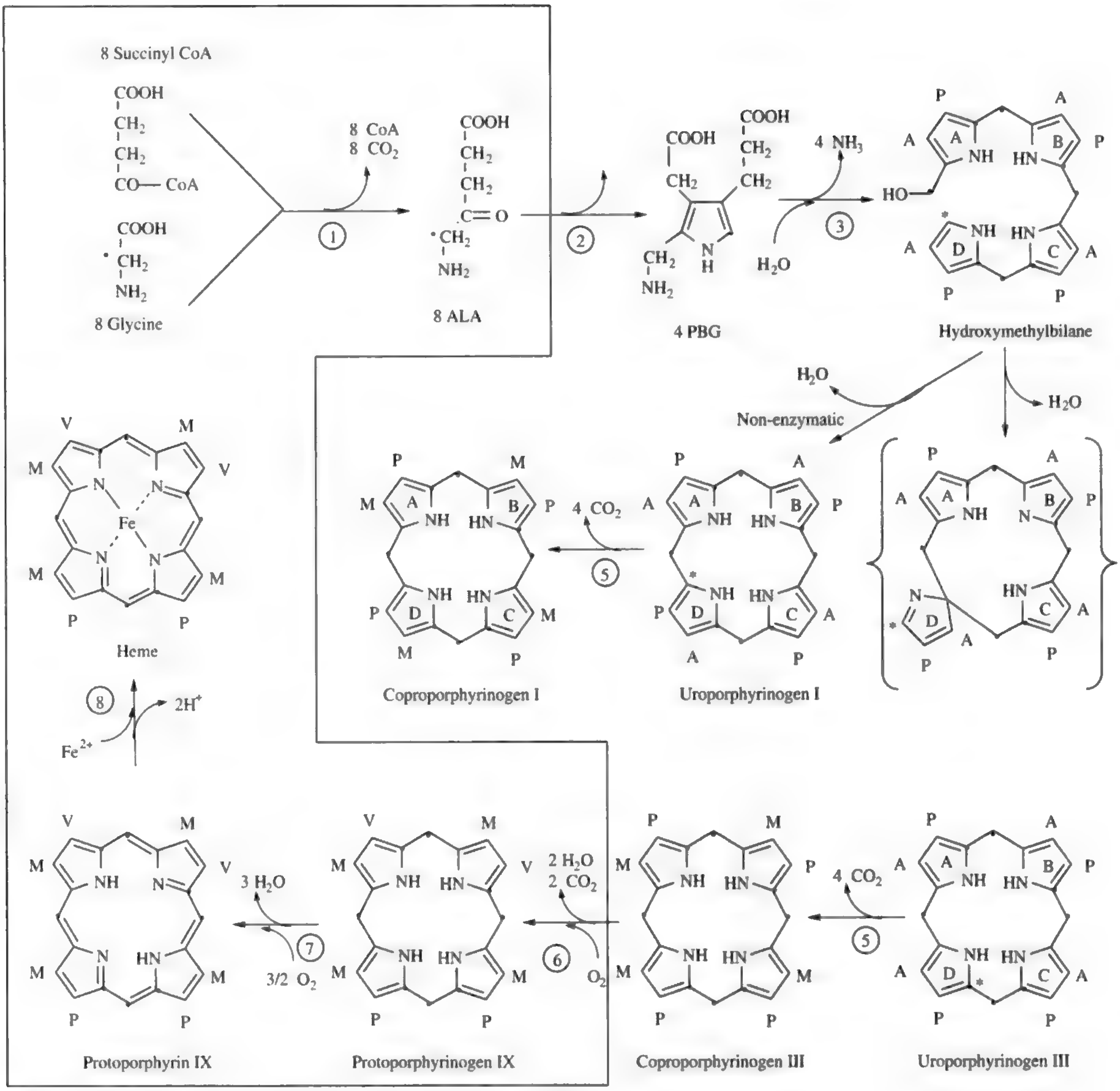
8 Succinyl CoA
8 Glycine
8 CoA
8 CO_2
8 ALA
4 PBG
4 NH_3
H_2O
Hydroxymethylbilane
Non-enzymatic
4 CO_2
Coproporphyrinogen I
Uroporphyrinogen I
Heme
$2H^+$
Fe^{2+}
3 H_2O
3/2 O_2
2 H_2O
2 CO_2
O_2
Protoporphyrin IX
Protoporphyrinogen IX
Coproporphyrinogen III
Uroporphyrinogen III
①
②
③
④
⑤
⑥
⑦
⑧

Fig. (2). The biosynthetic pathway for porphyrins and heme (68).

A; $-CH_2-COOH$; **M**; $-CH_3$; **P**; $-CH_2-CH_2-COOH$; **V**; $-CH=CH_2$

The carbon atom derived from the α carbon glycine is shown by a large point (•). The structure denoted by the brackets after step 4 is the presumed intermediate whose pyrrole ring D becomes rearranged to yield uroporphyrinogen III. At step 7 (protoporphyrinogen oxidase), 1 mol of oxygen is consumed per mol of water produced. (modified after N. Hayashi, *Protein, Nucleic Acid and Enzyme* 32:797-814, **1987** [160]).

human ALAS genes appear to have evolved by duplication of a common ancestral gene that encoded a primitive catalytic site, with subsequent addition of DNA sequences encoding variable functions [7]. The gene locus for the human ALAS-N is at chromosome 3p21 and that for the ALAS-E is at the Xp11.21 [6]. Inherited deficiency of ALAS-E is associated with X-linked sideroblastic anemia [8].

δ-Aminolevulinate Dehydratase (ALAD)

Two molecules of ALA are converted by a cytosolic enzyme, ALAD, to a monopyrrole, porphobilinogen (PBG), with the removal of two molecules of water (Fig. 2, step 2). ALAD deficiency porphyria (ADP) is due to an almost complete lack of enzyme activity. The human ALAD gene is localized at chromosome 9q34 [9]. The enzyme is a homooctamer with a subunit size of 36,274 daltons [10], and requires an intact sulfhydryl group and a zinc atom per subunit for full activity [11]. Lead inhibits ALAD by displacing zinc, the essential metal for enzyme activity, from the enzyme, resulting in neurological disturbances some of which resemble those of ADP [12]. The most potent inhibitor of ALAD is succinylacetone [13], a structural analogue of ALA which is found in urine and blood of patients with hereditary tyrosinemia who frequently develop a syndrome similar to ADP [14].

Porphobilinogen Deaminase (PBGD)

PBGD catalyzes the condensation of four molecules of PBG to yield a linear tetrapyrrole, hydroxymethylbilane (HMB). In the absence of the subsequent enzyme, uroporphyrinogen III cosynthase, HMB is spontaneously cyclized into the first tetrapyrrole, uroporphyrinogen I. In contrast, in the presence of the cosynthase, uroporphyrinogen III is formed which has an inverted D-ring pyrrole (Fig. **2**, step 3).

The gene locus for human PBGD is at chromosome 11q23→11qter [15]. The human PBGD gene is split into 15 exons, spread over 10 kb of DNA [16]. There are two isozymes of PBGD, i.e., erythroid-specific and non-specific [17]. The two isoforms of PBGD are produced by distinct mRNAs which are transcribed from a single gene by alternate transcription and splicing of its mRNA. A partial (or heterozygous) deficiency of PBGD is associated with acute intermittent porphyria (AIP).

Uroporphyrinogen III Cosynthase (UCS)

UCS catalyzes the formation of uroporphyrinogen III from HMB. This involves an intramolecular rearrangement which affects only ring D of the uroporphyrinogen (Fig. **2**, step 4). Human UCS predicted from its cDNA consists of 263 amino acid residues, with a molecular mass of 28,607 [18]. Homozygous deficiency of UCS is associated with congenital erythropoietic porphyria (CEP).

Uroporphyrinogen Decarboxylase (UROD)

UROD is a cytosolic enzyme which catalyzes the sequential removal of the four carboxylic groups of the carboxymethyl side chains in uroporphyrinogen to yield coproporphyrinogen (Fig. **2**, step 5). Human UROD cDNA consists of 10 exons which are spread over 3 kb [19]. The gene for UROD has been localized to chromosome 1pter→p21 [20]. Porphyria cutanea tarda (PCT) is due to a partial (or heterozygous) deficiency of UROD, while hepatoerythropoietic porphyria (HEP) is due to a homozygous deficiency of the enzyme.

Coproporphyrinogen Oxidase (CPO)

CPO is a mitochondrial enzyme which catalyzes the removal of the carboxyl group and two hydrogens from the propionic group of pyrrole rings A and B of coproporphyrinogen III to form a vinyl group at these positions, resulting in protoporphyrinogen IX (Fig. **2**, step 6). Unlike earlier steps in the biosynthetic pathway, only the type III coproporphyrinogen is utilized by CPO as substrate. The gene for human CPO is localized to chromosome 9 [21]. Human CPO predicted from its cDNA is a protein of 354 amino acids residues [22]. Hereditary coproporphyria (HCP) is due to a partial (or heterozygous) deficiency of CPO.

Protoporphyrinogen Oxidase (PPO)

The oxidation of protoporphyrinogen IX to protoporphyrin IX is mediated by a mitochondrial enzyme, PPO, which catalyzes the removal of 6 hydrogen atoms from the porphyrinogen nucleus (Fig. **2**, step 7). Variegate porphyria (VP) is due to a partial (or heterozygous) deficiency of PPO. Recently, cDNA and the structure of the human PPO gene has been reported [161,162]. The cDNA encodes for a protein with 477 amino acids with a calculated molecular mass of 50.8 kDa [161]. The promoter region contains multiple Sp1 elements, CCAAT boxes, and potential GATA-1 binding sites. There are the same transcripts expressed both in erythroid and in nonerythroid cells [162].

Ferrochelatase (FC)

The final step of heme biosynthesis is the insertion of iron into protoporphyrin (Fig. **2**, step 8). This reaction is catalyzed by a mitochondrial enzyme, FC. Unlike other steps in the heme biosynthetic pathway, this enzyme utilizes protoporphyrin IX as substrate, rather than its reduced form. However, the enzyme specifically requires ferrous, but not ferric, iron. The gene for human FC has been assigned to chromosome 18q21.3 [23]. The human FC gene contains 11 exons and has a minimum size of ≈ 45 kb [23]. Erythropoietic protoporphyria (EPP) is due to a partial (or heterozygous) FC deficiency.

Control of Heme Synthesis in the Liver and Erythroid Cells

Biosynthesis of heme in the liver is controlled largely by the rate of formation of ALAS [4], i.e., ALAS-N (Fig. **3**). The enzyme activity in normal liver cells is very low, while its level increases dramatically when the liver is required to synthesize more heme in response to various chemical treatments [24]. The synthesis of the enzyme is also regulated in a feedback fashion by heme, i.e., the end-product of the biosynthetic pathway [25]. There are at least 4 sites at which heme may exert a suppressive effect on ALAS-N [26]. Namely, heme has been shown to repress the transcription of ALAS-N gene expression [24,25,27], to decrease translation of ALAS-N [24,25,27], to interfere with the transfer of the neo-synthesized ALAS-N into mitochondria [28] and to inhibit its activity [29] (Fig. **3**). At higher heme concentrations than those which repress the synthesis of ALAS-N, heme induces microsomal HO, resulting in an enhancement of its own catabolism [25]. It can be thus visualized that hepatic heme concentration is maintained by a balance of the synthesis of ALAS-N and HO, both of which are under the regulatory influence of heme. In contrast, ALAS-E synthesis in erythroid cells is either refractory to, or is stimulated by heme treatment, or is developmentally increased when heme content increases in the cell (Fig. **3**). For example, heme has been shown to stimulate ALAS-E mRNA transcription [30], and to increase translation of ALAS-E mRNA [31]. Thus regulation of heme synthesis in erythroid cells is different from that in the liver [32].

Pathophysiology of Porphyrins and Their Precursors

Photosensitivity

Photosensitivity in cutaneous forms of porphyria is due to the accumulation of free porphyrins in the skin. Free porphyrins occur normally only in small amounts, but their levels may become markedly elevated in the porphyrias. Upon illumination at wavelengths ≈ 400nm (Soret band) and in the presence of oxygen, porphyrins cause photodynamic damage to tissues, cells, subcellular elements and biomolecules via the formation of singlet oxygen [33].

Neurologic Disturbances

Acute hepatic porphyrias, i.e., ADP, AIP, HCP and

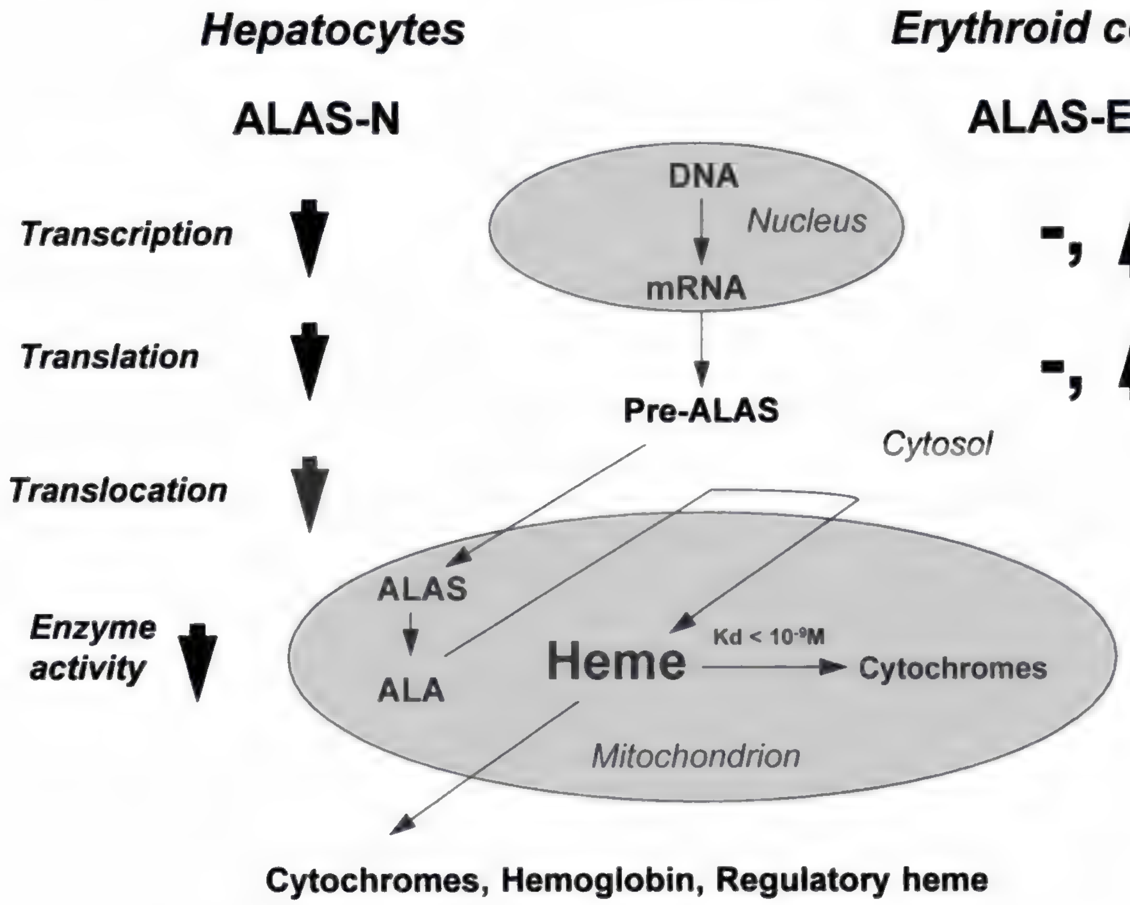

Fig. (3). The role of heme in the regulation of ALAS-N and ALAS-E gene expression.

ALAS-N in the liver is suppressed at 4 different levels by heme. In contrast, ALAS-E expression in erythroid cells is either refractory to, or upregulated by heme during their cell differentiation.

VP, are characterized by neurologic disturbances. Unlike erythropoietic porphyrias, these hepatic porphyrias are also accompanied by excessive production and excretion of porphyrin precursors such as ALA and/or PBG with the one exception of ADP which excretes excessive ALA, but not PBG. The most common symptoms associated with hepatic porphyrias are abdominal pain, disturbances in intestinal motility (e.g., diarrhea and constipation), dysesthesias, muscular paralysis, and respiratory failure which can often be fatal. ALA administration to normal animals does not cause any pathological consequence. Despite the fact that a number of theories exist, the exact nature of the neurological disturbances in the porphyrias remains obscure [26]. Extreme heme deficiency encountered in the subset of homozygous patients of the autosomal dominant porphyrias such as VP is accompanied by various developmental anomalies and abnormal physiology, e.g., loss of resistance to stress, numerous epileptic seizures, and developmental defects such as dwarfism or clinodactyly [34-37].

Four Major Applications of Heme and Heme-Related Compounds

Heme and Heme-Analogues in the Treatment of Porphyrias and Hematological Disorders

The porphyrias are a group of disorders caused by deficiencies in the activities of the enzymes of the heme biosynthetic pathway. The enzyme deficiencies can be either partial, or nearly complete. As a result, abnormally elevated levels of porphyrins and/or their precursors, e.g., ALA and PBG, are produced in excess, accumulate in tissues and are excreted in urine and stool. The two major symptoms of the porphyrias are cutaneous photosensitivity and neurologic disturbances.

Heme was used in the treatment of AIP for the first time some 20 years ago [38]. Since then, it has been recognized as the most potent and specific form of treatment for acute porphyria attacks [39]. The rationale for heme treatment is based on its repressive effect on the expression of hepatic ALAS, i.e., ALAS-N [25,26]. Overexpression of ALAS-N has been shown in patients with AIP during acute attacks [40,41], and in chemically induced hepatic porphyrias in animals [42,43], which is further exaggerated by presumed hepatic heme deficiency [26]. Heme infusion increases heme concentration and reduces porphyrin concentration in bile in man [44], suppresses the overexpression of ALAS-N, as directly shown in experimental porphyrias [45,46], and indirectly by normalization of excessive excretion of porphyrin precursors in patients with acute attacks of porphyrias [39,47-50]. Heme infusion also partially restores decreased mixed function oxidase activities and corrects clinical abnormalities [51,52]. Intravenous hematin at 4 mg/kg body weight given every 12 hours has been shown to be effective in reducing ALA and PBG excretion as well as in curtailing acute attacks [39].

The side effects of hematin infusion are unique alterations in hemostasis, and thrombophlebitis (Table II). Hemostasis abnormalities include prolongation of the prothrombin and thromboplastin times, an increase in fibrinogen degradation products, and thrombocytopenia [53,54]. *In vitro* studies have shown that hematin causes activation of a Factor XII-dependent pathway in human plasma [55], marked morphologic alterations in endothelial cell cultures, and increased platelet adhesion to a monolayer of cells [56], which may be in part responsible for the pathogenesis of thrombophlebitis observed during infusion. These side effects appear to be due to a degradation product(s) which occurs in hematin preparation, since they are more commonly seen with decayed solutions of hematin than with freshly prepared solution [57].

Table II. Side Effects of Hematin

		References
In vivo	Thrombophlebitis,	[53,54]
	Prolongation of prothrombin and thrombin times,	
	An increase in fibrinogen degradation products,	
	Thrombocytopenia	
In vitro	Activation of Factor XI-dependent pathway	[55]
	Marked alterations in morphology of endothelial cells in culture	[56]
	Platelet adhesion to monolayers	[56]

Heme arginate [58] is a stable form of heme which is easier to use than the conventional hematin solution. The liquid formulation of heme arginate consists of hemin (25 mg/ml), with 4 molar equivalents of arginine in propylene glycol:ethanol:water (4:1:5). The stock solution of heme arginate was reported to be stable for 2 years at 6°C [58]. While thrombophlebitis was commonly seen at the site of injection of hematin in animals and men, heme arginate did not cause any thrombophlebitis even after repeated injections [58]. Upon dilution in serum, heme dissociates readily from arginine and is available to bind to albumin. Thus heme arginate becomes essentially identical to heme-albumin which can regulate ALAS-N and HO-1 in the liver [58]. Synthetic heme analogues, e.g., Sn-mesoporphyrin, which inhibit heme catabolism and result in an increase in the level of regulatory heme [59], have also been shown to diminish the output of ALA, PBG and/or porphyrins in AIP and VP patients [60].

Heme arginate has also been used to treat patients with certain hematological disorders. The rationale of the use of heme in hematology is based on the stimulatory effect of heme on hemoglobin synthesis in murine erythroleukemia cells [61], induction of erythroid maturation of leukemic cell lines [62], and stimulation of the growth of primitive erythroid progenitor cells *in vivo* [63]. The drug was infused at 2 - 3mg heme/kg body weight weekly for 8 to 12 weeks, to 29 patients with myelodysplastic syndrome except chronic myelogenous leukemia who represented all French-American-British types [64,65]. While the majority of patients showed no response, the cytopenia improved in 6 patients, and returned to normal in three of these patients.

Four patients in 2 families with hereditary sideroblastic anemia were also treated with heme arginate [66]. No effect was observed on the mildly anemic hemoglobin levels or on the red cell counts, however, the abnormal myeloid to erythroid ratio in the marrow improved in all patients. In one family, abnormal heme synthetic enzyme activities were normalized, while there were consistent effects in the other family. Thus heme arginate may be useful as a new mode of treatment of certain hematological conditions, but further studies are clearly needed to establish this point.

Synthetic Metalloporphyrins in the Treatment of Hyperbilirubinemia

Neonatal hyperbilirubinemia is a common medical problem, which is in part due to the increased production of bilirubin from the catabolism of fetal hemoglobin, and in part due to the poorly developed capacity to excrete bile pigments in the newborn. In normal healthy full-term neonates, the plasma bilirubin concentration transiently rises during the first few days of life, then returns to the normal range by the second week of life. Some infants, however, show marked increases in plasma bilirubin during the immediate postnatal period; 16% reach peak bilirubin levels greater than 10 mg/dl, and 5% greater than 15 mg/dl [67]. Extended and exaggerated bilirubin levels in the newborn result in serious toxicity, termed "*kernicterus*", which is characterized by progressive lethargy, hypotonia, muscular rigidity, opisthotonos, spasticity, high-pitched cry, fever, convulsions, and ultimately death [68]. The two conventional treatments of neonatal hyperbilirubinemia presently in use are phototherapy and exchange transfusion. Phototherapy, treatment of choice, is based on photocatalysis of bilirubin isomerization which produces a more polar product, and is highly effective in lowering plasma bilirubin concentrations in infants with unconjugated hyperbilirubinemia [68]. More severe forms, or other types of unconjugated hyperbilirubinemia, e.g., Rh erythroblastosis, which are at risk of kernicterus are treated with exchange transfusion in neonatal cases [68].

Two inhibitors of HO-1, Sn-protoporphyrin IX (Sn-PP) and Sn-mesoporphyrin IX (Sn-MP), have been studied at the clinical level with respect to their ability to lower plasma bilirubin levels [69-71]. Subjects of study have included normal volunteers, patients with various forms of jaundice, a newborn infant with Crigler-Najjar type I syndrome [72], and a group of full-term newborns with jaundice associated with direct Coombs'-positive ABO incompatibility [70]. The results indicated that inhibitor treatment diminished the incremental changes in plasma bilirubin levels at all time points studied after birth, with the effects being statistically significant at 48 hours and thereafter in infants receiving a total of either 1.5 μmol/kg, or 2.25 μmol/kg. The only side effect noted was transient erythema while disappearance without sequelae during concurrent phototherapy with broad-spectrum lights. The overall requirement for phototherapy was diminished by about 40% in the combined treatment groups [70]. Sn-MP, presently the compound of choice, is a dose-dependent manner significantly ameliorated the course of hyperbilirubinemia in newborns of gestational age 210 - 251 days. At the highest dose of Sn-MP, the mean peak plasma bilirubin concentration was reduced by 41% and phototherapy requirement was decreased by 76% [71].

Sn-PP has also been tried in the treatment of 10 patients with psoriasis, in conjunction with long wave length ultraviolet (UVA) light. Psoriatic lesions were improved in all patients and the effect was reported striking in some [73]. In this case, the photochemical property, rather than the HO inhibitor activity, of the porphyrin appears to be responsible for its clinical efficacy.

Porphyrins in Photodynamic Therapy of Malignant Tumors

Photodynamic therapy (PDT) has been widely used in the treatment of a variety of malignant tumors. The principle of PDT using porphyrins is based on both the tumor localizing and photosensitizing properties of hematoporphyrin (HP), its derivative (HPD), or its active fraction, Photofrin II [74,75]. When injected in aqueous solution, HP and HPD selectively distribute to neoplastic tissues over normal tissues of the same cell type, though the exact mechanism for selectivity is unclear. Selectivity was demonstrated with respect to both amount and kinetics in cultured HepG2 cells [76]. However, when injected in mice, the amount of [^{3}H]HPD or [^{14}C]HPD which localized in the transplanted tumor at various times following i.p. injection (10 mg/kg) was higher than in skin or muscle, but was less than in liver, kidney, or spleen [77]. Thus the selectivity is rather weak, and should not be taken as a general rule.

Photofrin II is the commercial name for the active fraction of HPD and is thought to be an aggregated mixture of HP molecules linked by either ether or ester bonds [78,79]. Photofrin II, or HPD is an excellent photosensitizer *in vitro*, but only a moderate photosensitizer *in vivo*, probably because there is significant attenuation of light penetration in tissues. The attenuation of light in tissues is known to decrease with increasing wavelength [80]. While light at 630 nm is usually used in PDT to minimize tissue absorption, a new photosensitizer which can be activated by light of longer wavelength is clearly more ideal. Optical penetration depths (defined as the depth at which the incident light intensity is reduced to 37% of its initial value) are usually < 1 mm for wavelengths of light below 600 nm and approximately 2.5 mm to 4.5 mm for light above 630 nm [81].

Singlet oxygen ($^1O_2$) production via a type II photochemical reaction is believed to be mainly responsible for cellular and tissue damage induced by PDT [82], although other oxygen radicals are also produced [83,84]. The first clinical trial of PDT was conducted in 1976, and more than 3,000 patients have since been treated with PDT [85]. The primary side effect is skin photosensitization. The most encouraging results have been obtained with superficial tumors, such as solid tumors of bronchus, esophagus, bladder, treated with the aid of fiberoptics, and head, neck, skin and eye by direct irradiation [85].

PDT, using dye-mediated photosensitization, has been recently studied as a new mode of treatment in bone marrow transplantation. Four photosensitizers have been evaluated as potential purging agents of marrow grafts contaminated with neuroblastoma and/or leukemia and lymphoma cells; merocyanine 540 (MC540) [86-91], dihematoporphyrin ether (Photofrin II) [92], pyrene-containing fatty acids [93], and sulfonated chloraluminum phthalocyanine [94]. Experiments with tumor transplantation and established tumor cell lines have shown that MC540-mediated photosensitization kills leukemia, lymphoma, and neuroblastoma cells much more rapidly than primitive normal hematopoietic progenitor cells [86,89-91,95]. Photofrin II, however, appears to be not more effective than MC540 against solid tumors [96].

As a variation of porphyrin-based tumor therapy, a boronated metalloporphyrin was used in boron neutron capture therapy [97]. Using $^{10}B_1$-paraborono-phenylalanine ($^{10}B_1$-BPA) in boron neutron capture therapy, several human patients with malignant melanoma have been successfully treated [98]. The application of ($^{10}B_1$-BPA) in boron neutron capture therapy is, however, complicated by several factors; e.g., a large total dose must be given since the weight percentage of boron in BPA is only about 5%, BPA has limited solubility at physiological pH, and the dihydroxyl boryl group of BPA is not hydrolytically stable under physiological conditions [97]. A manganese boronated protoporphyrin (Mn-^{10}BOPP) when tested in murine melanoma and angiosarcoma cells has been shown to be more tumor selective, and possess greater tumor killing activity than $^{10}B_1$-BPA [97].

Porphyrins as Contrast Reagents in Radiography

A synthetic metalloporphyrin, gadolinium (Gd) (III)-tetraphenylporphine sulfonate (Gd-TPPS) [99], has been successfully used as a contrast agent for *in vivo* magnetic resonance imaging (MRI) in rat brain glioma. After injection of Gd-TPPS, the signal intensity of experimental rat brain glioma distinctly increased on T1-weighted MRI, an effect similar to that produced by the clinically applied MRI contrast agent, Gd-diethylenetriaminepentaacetic acid (Gd-DTPA). Mn-mesoporphyrin incorporated into liposome membrane containing oleic acid was examined as an oral MRI contrast agent of the gastrointestinal tract [100], and Mn-uroporphyrin as an intravenous MRI contrast agent for brain tumors [101]. While porphyrins are considered as possible endogenous ligands for peripheral-type benzodiazepine receptors [102], the tumor-specific enhancement of Mn-TPPAS is not mediated by the benzodiazepine receptor [103,104].

The Role of Heme in Gene Expression

Recent evidence indicates that there are significant tissue-specific expression and heme-mediated regulation of enzymes in the heme biosynthetic pathway. As discussed above, hepatic (ALAS-N) and erythroid ALAS (ALAS-E) are isozymes which are encoded by two separate genes. ALAS-N expression in the liver is under negative control by heme, while in the developing erythroblasts, ALAS-E gene expression is positively influenced by heme. It is also known that iron metabolism in erythroid cells, as compared with non-erythroid cells, is regulated in a distinct manner [105]. In addition to the distinctive control of ALAS expression in these tissues, ALAS in the rat Harderian gland was found to be the ALAS-N isoform and yet not influenced by heme [106]. Thus at least three different types of regulation of ALAS by heme are known.

In addition to the tissue-specific regulation of heme biosynthesis, heme has also been shown to influence a number of gene functions which are not directly related to heme synthesis in various tissues. Possible effects of heme on various cellular functions are summarized in Fig. **4**. As shown in this figure, heme serves as the prosthetic group for hemoproteins; downregulates and upregulates ALAS-N in the liver and ALAS-E in erythroid cells, respectively; increases NF-E2 binding activity in erythroid cells [106]; is an essential part in the inducible nitric oxide synthase complex in NO production [107]; plays an important role in cGMP production since soluble guanylate cyclase is a heme-containing enzyme and its activation by NO possesses an absolute requirement for enzyme-bound heme [108]; serves as the substrate for HO resulting in the production of CO, biliverdin and iron [109]; upregulates HO-1 gene expression [109]; inhibits phosphorylation of eIF2α by its kinase and as a consequence sustains protein synthesis [110] and stimulates growth and cell differentiation of certain cell types [111].

Other Applications

Heme and porphyrins have also other potentially useful actions in various systems *in vitro*. Hemin was found to enhance the antiviral action of 3'-azido-3'-deoxythymidine (AZT) against both drug-resistant and drug-sensitive human immunodeficiency virus type-1 (HIV-1) strains [112]. The effect was more pronounced against the latter. Hematin alone was also able to inhibit viral replication in concentrations significantly smaller than those required to inhibit reverse transcriptase (RT) activity of Rauscher murine leukemia virus. A series of cationic metalloporphyrin-ellipticine complexes were also found to inhibit the cytopathicity of HIV-1 and simian immunodeficiency virus in cell cultures [113]. These compounds were also found to inhibit syncytium formation between persistently HIV-1-infected cells and uninfected cells, to interfere with HIV-1 binding to the cells, and to suppress HIV-1-associated RT activity [113]. Despite these findings, it remains unclear how these compounds inhibit virus-cell binding. Inhibition of RT activity may be mediated solely by interference with the viral adsorption step, if it occurs. The effect apparently does not require light activation; thus it may be related to the property of certain porphyrins to intercalate into nucleic acids, as described below.

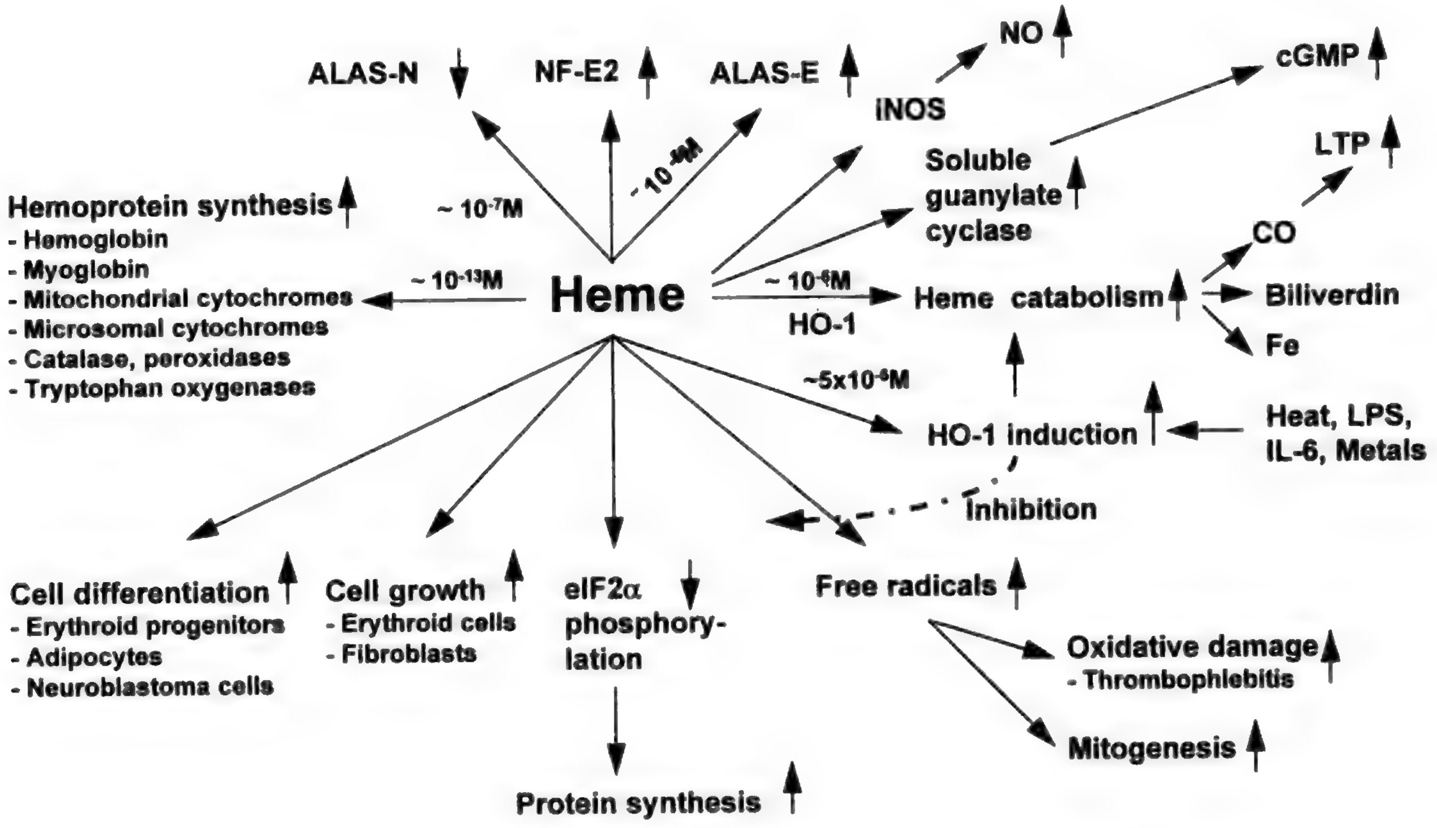
ALAS-N ↓
NF-E2 ↑
ALAS-E ↑
NO ↑
INOS
cGMP ↑
Soluble guanylate cyclase ↑
LTP ↑
Hemoprotein synthesis ↑
- Hemoglobin
- Myoglobin
- Mitochondrial cytochromes
- Microsomal cytochromes
- Catalase, peroxidases
- Tryptophan oxygenases
~ 10^{-7}M
~ 10^{-6}M
~ 10^{-13}M
Heme
~ 10^{-6}M
HO-1
Heme catabolism ↑
CO
Biliverdin
Fe
~$5x10^{-6}$M
HO-1 induction ↑
Heat, LPS, IL-6, Metals
Inhibition
Cell differentiation ↑
- Erythroid progenitors
- Adipocytes
- Neuroblastoma cells
Cell growth ↑
- Erythroid cells
- Fibroblasts
eIF2α ↓ phosphorylation
Free radicals ↑
Oxidative damage ↑
- Thrombophlebitis
Mitogenesis ↑
Protein synthesis ↑

Fig. (4). Effects of heme on various cellular functions.

Heme has both positive and negative influences on various cellular functions.

Cationic porphyrins have high binding constants to DNA and exhibit uniquely diverse binding modes which are dependent both on the porphyrin species and the DNA. Not only do these porphyrins bind electrostatically outside at AT base pairs [114], but many appear to be quite capable of sliding their bulky ring system between GC base pairs via intercalation [115,116]. Tetracationic *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin (TMpyP) (Fig. **5**) and its metalloderivative (MTMpyP) have received the most study [114]. The high DNA binding property of these porphyrins may in part be responsible for their tumor-localizing effect. Upon binding to DNA, TMpyP causes sister chromatid exchanges in *Allium cepa* root cells [117,118]. In contrast, HPD does not appear to cause chromatin damages as does TMpyP [117]. These synthetic metalloporphyrins have several advantages as model drugs and probes of nucleic acids, since they are quite soluble in water and their solution chemistry can easily be studied, they are either intense chromophores or markedly fluorescent, making their detection simple, and the majority of these cationic porphyrins do not aggregate, simplifying analysis, and chemical modification of their structure can readily be made [119].

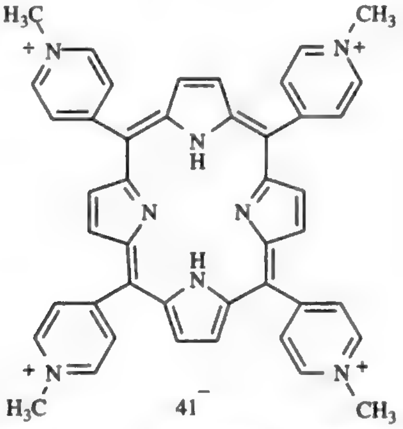

Fig. (5). Structure of Tetracationic *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin (TMpyP) [118].

Biodistribution

Biodistribution is an important consideration in the application of heme and heme-related compounds. Heme is rather insoluble in aqueous solution at physiological pH, and is highly aggregated [120]. Hematin dimers form as a result of the formation μ-oxo dimers (Fe-O-Fe) with loss of a water molecule from two hematin molecules. Thus under physiological conditions, e.g., in plasma, essentially all heme is present as protein-bound. This is also true for protoporphyrin, however, other porphyrins such as coproporphyrins and particularly uroporphyrins are significantly more soluble in water. Thus protoporphyrin is excreted almost exclusively into bile and stool, while uroporphyrin is excreted into urine. On the other hand, coproporphyrin can be found both in stool and urine, reflecting its intermediate hydrophobicity between that of protoporphyrin and uroporphyrin. The majority of heme and protoporphyrin in plasma, which is usually a very small fraction compared with their concentrations in the cell, e.g., erythrocytes, is bound to albumin with a dissociation constant (K_d) of ≈ 10^{-8}M [121]. Kd's of albumin for other porphyrins are also in the similar range, e.g., protoporphyrin (Kd=10^{-6}M - 10^{-7}M), HP (Kd=10^{-6}M), and Sn-PP (Kd=10^{-5}M - 10^{-6}M). The binding of heme and porphyrins to albumin can occur at one or several different domains; the high affinity site of albumin binds hemin as the monomer [121], while the lower affinity sites may bind porphyrins both as monomers and dimers [122,123].

Hemopexin is a plasma glycoprotein with a molecular weight of 57,000, which has a single affinity site for heme (Kd=10^{-13}M) [124]. There are two histidines at this site which serve as fifth and sixth ligands for heme. While hemopexin is less abundant than albumin (1.5% - 3% of albumin), > 95% of exogenously administered heme is transferred from albumin to hemopexin within several hours [125]. Hemopexin also binds other porphyrins, but with much less affinity than heme. For example, metal-free porphyrins bind with hemopexin, with Kd ≈ 10^{-6}M, similar to albumin [124,126], while Sn-PP is efficiently bound with hemopexin (≈ 35%) at therapeutic concentrations of the drug [127].

Most exogenously administered hemin and porphyrins disappear from plasma within several hours, and are then found in the liver [128]. The plasma clearance of hematin in man is biphasic, with a half-life ($t_{1/2}$) of 11 ± 4 and 29 ± 9h for the initial and final phases, respectively [129]. Sn-PP is cleared from plasma with a much shorter $t_{1/2}$ of 4h, for plasma levels greater than 5 nmol/ml, with evidence of dose-dependent pharmacokinetics at lower plasma concentrations [130]. There is evidence that the exogenously administered hematin can enter the regulatory heme pool, repress the synthesis of ALAS-N, and saturate heme-dependent tryptophan pyrrolase activity [131]. After the heme moiety of hepatic cytochrome P450 in rats has been destroyed by allylisopropylacetamide, heme injected intravenously significantly increased the cytochrome P450 content and mixed function oxidase activity of liver microsomes, suggesting that exogenous heme can partially restore the structure and function of hepatic cytochrome P450 [132,133]. Exogenous heme is also effective in suppressing the overexpression of hepatic ALAS-N and partially correcting deranged hepatic mixed function oxidase activities during acute attacks in patients with acute hepatic porphyrias [26,39,51,52].

In addition to hemopexin and albumin, lipoproteins also bind porphyrins, particularly those which are hydrophobic. Lipoproteins are likely candidates for the transfer of the naturally occurring and therapeutically administered porphyrins into cells [134,135]. As much as 50% of porphyrin added to serum or fresh blood has been found to partition into lipoproteins [135]. Plasma of patients with porphyrias, or subjects injected with HP for photodynamic therapy also contains substantial amounts of lipoprotein-bound porphyrins [136,137].

The mechanism of hepatic uptake of heme is not clear. While a receptor for hemopexin has been suggested [138], it has not been isolated or characterized, and its role is also questioned along several lines of evidence [139,140]. There is evidence that, instead of serving as a carrier of heme to hepatocytes, it may serve as an acute phase protein to reduce the oxidant property of heme [141]. It has been shown that heme readily binds to lipid bilayer of liposomes, suggesting that heme can be transferred from a heme-albumin complex to the hepatocyte membrane. Several heme-binding cytosolic proteins which may serve as an intracellular heme transporter have been suggested in the liver [142,143], though their relative significance in the intracellular transfer of heme remains unclear.

Side Effects

Side effects of heme and porphyrins are summarized in Table II. Porphyrins are highly reactive species, and their photodynamic effects may severely damage cells and tissues. Porphyrins, on photoactivation, generate active oxygen species, e.g., superoxide anion, singlet oxygen and hydroxyl radical, that can cause lipid peroxidation and initiate membrane damage and other oxidative changes in the cell. Heme is also a pro-oxidant and cause lipid peroxidation *in vitro* [141,144]. Infusion of hematin solution is known to be associated with anticoagulant effects and phlebitis, and is believed to be the cause of oxidative damages to proteins and membranes [145]. The acute LD_{50}s are as follows; hemin (43 mg/kg, i.v., rats) [146]; heme arginate (56 mg/kg, i.v., mice) [58]; HPD (205 mg/kg, i.p., mice) [74]; and Photofrin II (130 mg/kg, i.p., mice) [74]. Toxicity for the latter two porphyrins, however, dramatically increases upon exposure of animals to light, i.e., 7.5 and 4 mg/kg, respectively. Sn-PP, at a dose greater than 100 mg/kg, can be administered to newborn rats subcutaneously, without significant adverse effects [147,148]. The oxidant properties of free heme is thought to play an important role in causing *in vivo* toxicity. Induction of HO-1 [149] and hemopexin [139,141] has been implicated as a defense reaction to curtail the toxic effects of heme, the former by destroying heme, the latter by binding it.

In contrast to heme, protoporphyrin appears to exhibit anti-oxidant properties, depending on the experimental systems. It has been shown that, in the dark, protoporphyrin inhibits lipid peroxidation of rat liver microsomes, while in the presence of light it may stimulate Fe^{3+}-ADP/ascorbate-induced lipid peroxidation [150]. Uroporphyrin has no such effect [150]. The inhibition of lipid peroxidation appears to be the result of the scavenging of peroxyl radicals by protoporphyrin. Other investigators showed that, in addition to protoporphyrin, MP and HP also inhibited lipid peroxidation [151].

Future Perspectives

More efficient and specific tumor-localizing photosensitizers need to be developed for use in PDT. Heme arginate is an excellent heme preparation, however, its specific delivery to target tissues remains to be clarified. The same applies to synthetic metalloporphyrins used in the treatment of hyperbilirubinemia. Synthetic hemes may be promising candidates as blood substitutes. It is important for drug development to develop valid methods of separation and quantification of porphyrins, metalloporphyrins and hemes. High performance liquid chromatography (HPLC) is usually the method of choice for determination of these compounds in biological samples [152], however, the poor solubility of some of these compounds may limit the use of HPLC, particularly that of reverse phase HPLC. Those compounds which are highly hydrophobic can be converted to the corresponding esters which can readily be separated by normal phase HPLC. The fluorescent property of porphyrins permits, often without separation by chromatography, very sensitive detection of these compounds in biological samples. Monoclonal antibodies for certain porphyrins such as Pd-coproporphyrin were prepared and may prove to be useful for specific quantification of these compounds [153].

Abbreviations Used

ADP = δ-Aminolevulinic acid dehydratase deficiency porphyria

AIP = Acute intermittent porphyria

ALAS = δ-Aminolevulinic acid synthase

ALAS-E = Erythroid-specific ALAS

ALAS-N = Non-specific ALAS

ALAD = δ-Aminolevulinic acid dehydratase

BPA = Boronophenylalanine

CEP = Congenital erythropoietic porphyria
CPO = Coproporphyrinogen oxidase
EPP = Erythropoietic protoporphyria
FC = Ferrochelatase
HCP = Hereditary coproporphyria
HEP = Hepatoerythropoietic porphyria
HMB = Hydroxymethylbilane
HO = Heme oxygenase
HP = Hematoporphyrin
HPD = Hematoporphyrin derivative
MP = Mesoporphyrin
PBGD = Porphobilinogen deaminase
PCT = Porphyria cutanea tarda
PDT = Photodynamic therapy
PP = Protoporphyrin
PPO = Protoporphyrinogen oxidase
TMpyP = Tetracationic *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin
TPPS = Tetraphenylporphine sulfonate
UROD = Uroporphyrinogen decarboxylase
UCS = Uroporphyrinogen III cosynthase
VP = Variegate porphyria

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Reduction of Biliverdins to Bilirubins: Its Metabolic Regulation Under Various Physiological Conditions

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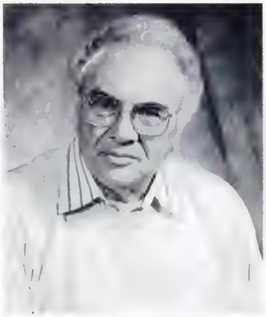

[1] Dedicated to the cherished memory of Rosalia B. Frydman, with whom we shared the challenge of the work reviewed in this article.

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Abstract: Heme and hemoproteins are degraded in mammals by oxidation to biliverdins. These linear tetrapyrroles are reduced to bilirubins by a cytosolic biliverdin reductase (BvR) at the rate of 250-400 mg per day. While the bulk of biliary biliverdin is biliverdin IXα, other isomers such as biliverdins IXβ and IXγ are formed under conditions of oxidative stress by the chemical degradation of hemoproteins, or from the degradation of abnormal hemoglobins. Rat liver BvR was found to be a NADPH-dependent reductase with a broad substrate specificity, which efficiently reduces a large number of biliverdins as long as they carry two propionate side-chains. The enzyme was found to exist in three molecular forms, two of which (molecular forms 1 and 3) interconvert under conditions of oxidative stress or in the presence of oxidant species. The different molecular forms have different reduction rates for the biliverdin isomers, thus securing the efficient reduction of biliverdins to bilirubins under different physiological conditions. The molecular mechanism of the enzymatic reduction entails the protonation of the basic pyrrolenine nitrogen (N23) which results in a mesomeric positive charge on the neighboring meso C-10 carbon. The C-10 then undergoes a nucleophilic addition of the hydride released by the NADPH cofactor of BvR. Our studies have established the structural requirements for a biliverdin to be efficiently reduced to a bilirubin. This metabolic step gains relevance as synthetic hemes and metalloporphyrins are increasingly used in therapeutics.

Biliverdins: Nomenclature, Occurrence, and Physiological Properties

Biliverdins are linear tetrapyrroles formed during the metabolic degradation of cyclic tetrapyrroles (cytochromes, hemoglobin, myoglobin). They form part of the rich history of bile pigment chemistry, and therefore retain the Fischer nomenclature of trivial bile pigment names [1]. This nomenclature can be combined with the more recent one [2] which favors the bilane **1** as the fundamental system (Fig. **1**).

For the dehydrogenated bilanes, the number of additional double bonds involving the linking of

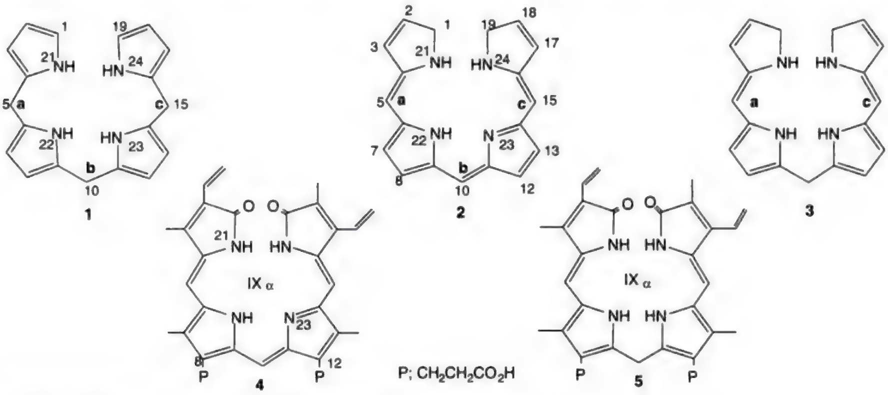

Fig. (1). Structures of bilane, bilatrienes, and biladienes.

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pyrroles are indicated by the letters *a, b, c* corresponding to the methine-positions 5, 10, and 15. Thus, biliverdin **2** is a bilitriene-*a,b,c* and bilirubin **3** is a biladiene-*a,c*.

The traditional Fischer nomenclature distinguishes isomers by taking into account the sequence of peripheral substituents of the parent porphyrin and assigning it a Roman numeral, followed by a Greek letter to indicate at which methine position the formal cleavage occurred. Thus, biliverdin IXα **4** is the trivial name of a bilitriene-*a,b,c*, while bilirubin IXα **5** is the traditional name of a biladiene-*a,c* (Fig. **1**).

Biliverdins are widely distributed in nature both as free compounds and as protein-bound chromophores. They are found in mammals, birds, amphibiae, fish, mollusks, and insects; as well as in algae, higher plants and anaerobic bacteria [3-6]. Biliverdin IXα **4** and its derivatives are the most widespread, but biliverdin IXγ **8** (pterobilin) (Fig. **3**) is also abundant either free or bound to proteins, in butterflies, choleopters, and insect hemolymph [7]. Biliverdin IXδ **6** and its derivatives are present in marine organisms [8]. Bactobilin, a unique biliverdin (urobiliverdin I) was isolated and identified in bacteria [9,10]. Biliverdin IXα **4** is formed in the enzymatic oxidation of heme (Fig. **2**) while biliverdins IXβ **7** and IXγ **8** (Fig. **3**) result from the chemical degradation of hemoproteins under conditions of oxidative stress. In humans, ca. 250-400 mg of biliverdin IXα **4** is produced daily and is reduced by biliverdin reductase (BvR) to bilirubin IXα **5**. This reduction is indeed surprising since biliverdins are water soluble and apparently inocuous, while bilirubins are highly hydrophobic and toxic compounds. They must be glucuronylated with expense of energy in order to be excreted. Since biliverdins are not reduced to bilirubins in invertebrates, birds, amphibiae, and fish (they are excreted without further structural modifications) the reason for this evolutive step in mammals is puzzling. Several explanations were advanced. One is that only bilirubin can pass through the membranes that separate the fetus from its mother, allowing for the excretion of the fetal heme degradation products [11]. This explanation is however, not valid for fish. It has also been proposed that bilirubin, which is transported tightly bound to serum albumin, is an important physiological antioxidant, and that the complex is a powerful scavenger of peroxyl radicals and of inhibitors of lipid peroxidation both in aqueous phase [12,13] and the lipid bilayer (at difference with other antioxidants such as glutathione and ascorbate [14,15]). However, biliverdins also seem to share the antioxidant properties of conjugated bilirubin [16,17] and also bind to albumin; hence, the question is far from solved.

Many interesting physiological roles have been attributed to the biliverdins; in hepatic regeneration [18], as inducers of proto-oncogenes in epithelial cells of rat liver [19,20], as tissue protectors against inflammatory damage [21], as antiviral agents against human herpes virus (HHV-6) [22], and as inhibitors of the cytopathic effects of HIV-1 [23]. Hyperbiliverdinemia has been detected in the bronze-baby syndrome [24], in patients affected by green jaundice [25,26] and in indomethacin induced hepatitis [27]. The chemistry and biological functions of linear oligopyrroles and bile pigments have been compiled and reviewed with great authority up to 1989 [30]; we had reviewed our early work on heme catabolism in 1987 [31]. The role of bilirubin in metabolism and disease is being constantly reviewed (110 reviews during 1992-1995), while the biological function of heme is reviewed in this issue [28], together with the photodynamic properties of porphyrins [29]. This article will therefore concern itself mainly with the work performed by our team during the last years on the chemistry and metabolic regulation of biliverdins.

Heme Catabolism

Heme (Fe^{2+}-protoporphyrin IX) is an essential metabolite involved in the synthesis of globin [32], the expression of the receptors of transferrin [33], the

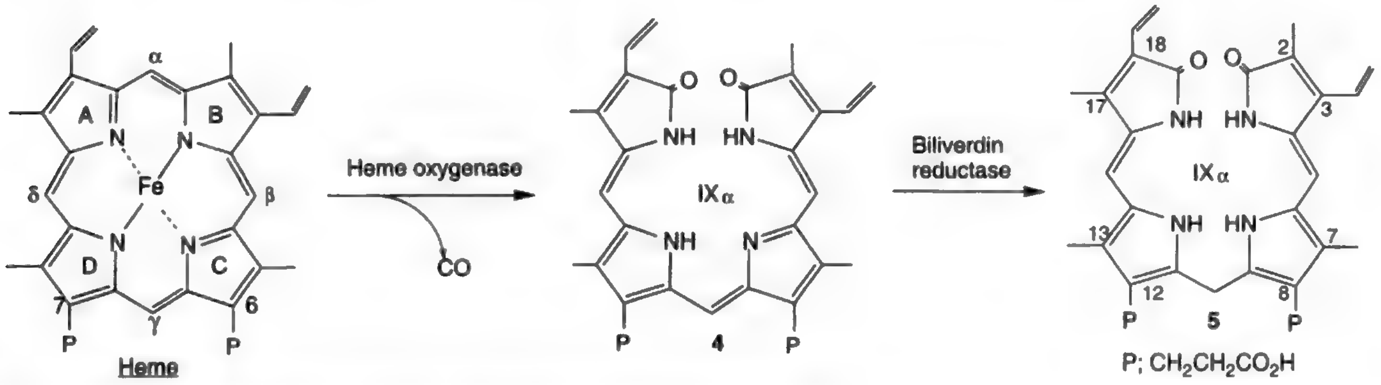

Fig. (2). Enzymatic conversion of heme to biliverdin IXα and bilirubin IXα.

regulation of the activities of guanylate cyclase [34] and adenylate cyclase [35], the inhibition of the ATP-dependent proteolysis [36] and the regulation of its own biosynthesis [37]. As the prosthetic group of hemoproteins it is involved in fundamental biological oxidation reactions with and without oxygen [38]. In mammals it is degraded by two enzymatic systems; a microsomal one, which comprises a heme oxygenase, and a cytochrome P450 (or cytochrome c) reductase that oxidize heme to give biliverdin IXα **4** [31]; and a cytosolic enzyme, biliverdin reductase (BvR) that reduces biliverdin IXα **4** to bilirubin IXα **5** (Fig. **2**). The latter is excreted after conjugation with glucuronic acid.

Extensive studies carried out by our team have shown: a) that heme oxygenase confers α-regioselectivity to the oxidation of heme, b) that only hemes which carry two propionate residues at C_6 and C_7 are enzymically oxidized, and c) that BvR is an enzyme with a broad substrate specificity that reduces bilitrienes-*a,b,c* to biladienes-*a,c* as long as the former carry two propionate residues in any of the eight peripheral pyrrole carbons, see (Fig. **2**) [31]. These *in vitro* results were confirmed by *in vivo* studies using perfusion techniques where bile fluids were collected after synthetic and natural hemins were perfused through rat liver [39,40]. At variance with the enzymatic oxidation of heme IX, its chemical oxidation (coupled oxidation) affords all the four possible isomers of biliverdin (IXα **4**, Xβ **7**, IXγ **8** and IXδ **6**, (Fig. **3**)). To keep the Fe^{2+} in its reduced state, a diaxial ligand (e.g., pyridine hemochrome, (Fig. **3**)) is used for the chemical oxidation.

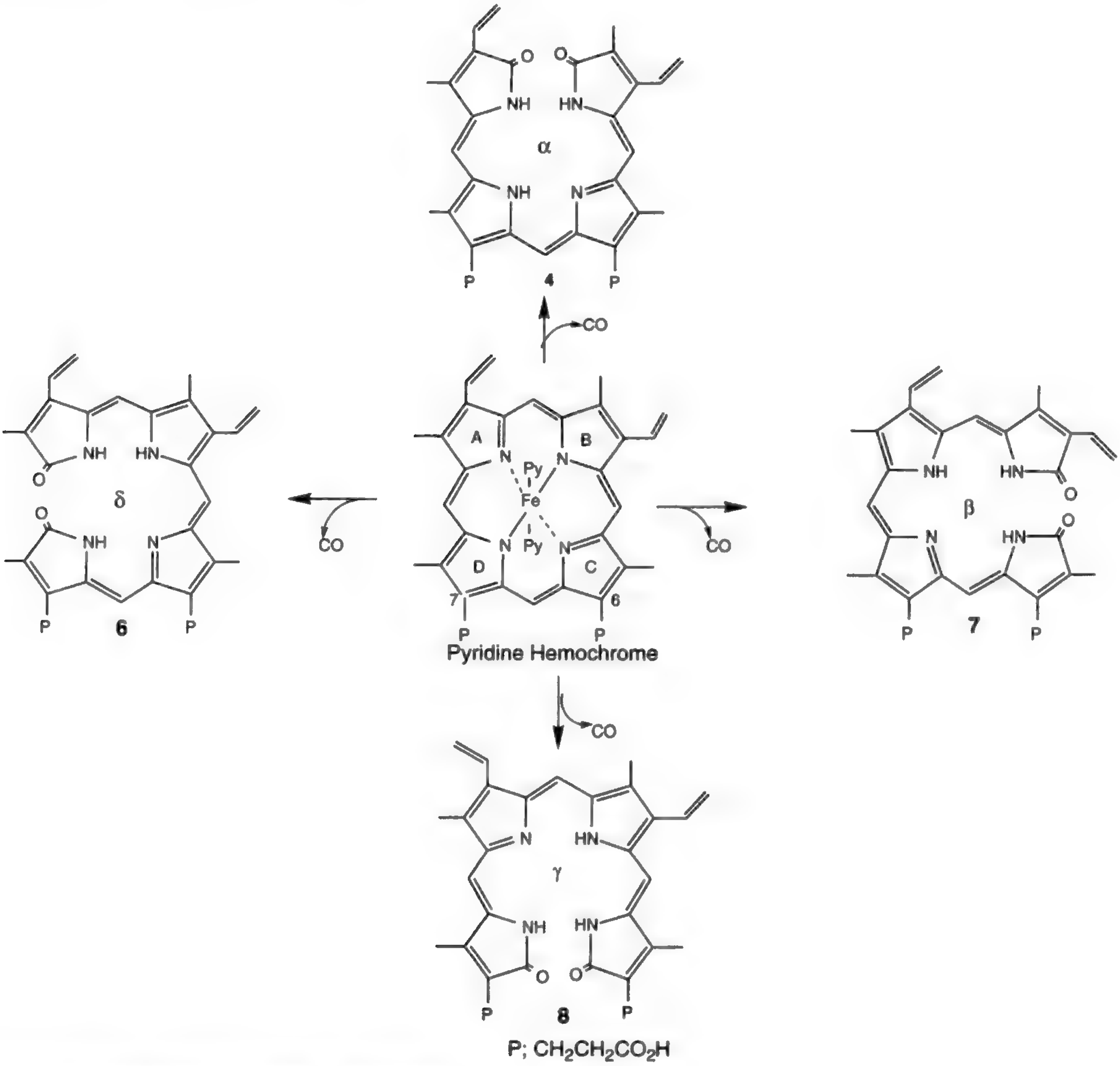

Fig. (3). Chemical oxidation of heme to biliverdins.

If hemoproteins are used as substrates for the chemical oxidation, the axial ligands may be provided by the basic amino acid residues of the protein. It is the protein that then introduces regioselectivity by shielding meso-positions (5,10,15 or 20) from the intramolecular attack of the dioxygen ligand in the axial position. This is shown by the exclusive formation of biliverdins IXα **4** and IXβ **7** in the oxidation of catalase [41] and hemoglobin [42], as well as in the significant formation of biliverdin IXγ **8** during the oxidation of hemoglobin-Zurich [43]. The chemical oxidation of hemoproteins might be operative *in vivo* too, under conditions of oxidative stress [44] (see below).

The conformation of biliverdins

In a linear tetrapyrrole system such as that found in biliverdin IXα **4** an overall conformation resembling a helical arrangement becomes possible. Thus, an octa-substituted biliverdin such as **9** (Fig. **4**) has an overall helical arrangement with a distance (pitch) between O1 and O19 of 3.25Å. The system is held together due to an efficient intrachromophoric hydrogen bond system based on the N24-H•••N23•••H-N22 bonding network.

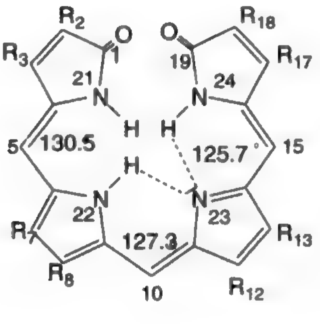

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Fig. (4). Intrachromophoric hydrogen bond system in biliverdin.

Note the difference between the degrees of the bonding angles at C_5 (130.5°) and C_{15} (125.7°) which is due to a hydrogen bridge between N24-H and N23. The distances of the intramolecular bonding system can be seen in (Fig. **5**) [30].

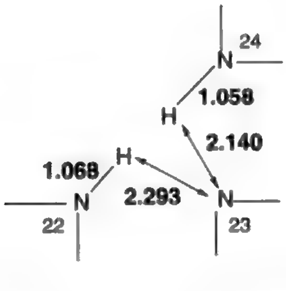


Fig. (5). Distances (Å) of the intramolecular hydrogen bonding system in biliverdin crystals.

The chromophore adopts a (4Z, 9Z, 15Z) configuration and an all-syn periplanar conformation that may be termed helical. If, on looking down the axis of such a helix, one has to proceed in a clockwise manner to reach the most distant part, the absolute sense of the helix is assigned to (P) (from *plus*); if the contrary is the case, the symbol is (M) (from *minus*) (Fig. **6**). The (P) and (M) enantiomers of a helical biliverdin resemble "locker washers."

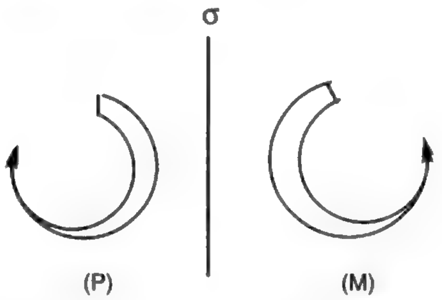
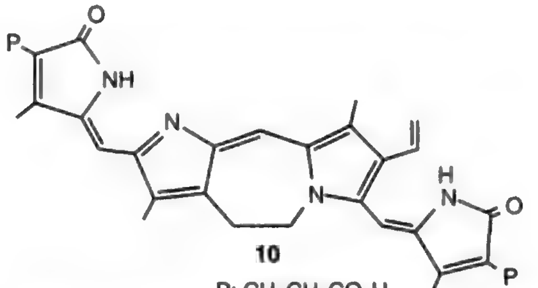


Fig. (6). "Locker-washer" conformational enantiomers of a helical biliverdin.

A striking crystallographic confirmation of the above mentioned conformations was obtained when the X-ray analysis of the crystals of the bilin binding protein from the cabbage male butterfly (*Pieris brassicae L.*) was measured [45]. Its prosthetic group is biliverdin IXγ **8** (pterobilin). The overall shape of the protein resembles a calyx and the IXγ- chromophore is located (non-covalently attached) in the central cleft at the upper end of the barrel. Its configuration within the protein is (Z,Z,Z); that it is a right-handed helix (P) was established in an absolute sense by correlation with the conformation of the protein. Its CD spectrum allowed to secure its configuration in agreement with the sign rule for C_2-symmetrical chromophores [46]. These results put the conformational studies of helical biliverdins on a firm basis. Each helical biliverdin is a 1:1 mixture of the (P) and (M) enantiomeric forms. Recombination with a regioselective protein pocket allows the separation of both forms. Thus, on recombination of biliverdin IXα **4** with apomyglobin, only the (P) conformation was found to bind into the heme pocket [47,48]. We will come back to these results when discussing the enzymatic reduction of biliverdins.

A disruption of the efficient intrachromophonic hydrogen bond system shown in (Fig. **4**) drives the tetrapyrrole chain into an extended conformation; a geometry that can be achieved under different experiemental conditions. Whereas N-methylation at N21 and N24 in the bilitriene does not affect the overall helical conformation, the N23-methyl derivative adopts a partially extended conformation [49]. If compensation for the N23•••H-N24 hydrogen bond is provided by other optimally oriented donor groups such as those present in certain covalently bound biliverdin-tripeptides, the tetrapyrrole chain goes into an extended conformation [50], very likely by rotation around the C14-C15 bond. Protonation of the pyrrolenine nitrogen (N23) has a similar effect [51]. In the monoprotonated N$^+$23-H species, the central hydrogen bond system is destroyed and the helical conformation changes to a more extended one, by rotation around the C9-C10 bond. The "stretching" of the helical conformations when saline effects and donor groups compensate for the intramolecular hydrogen bond system is to be found in the plant and algae biliproteins. Binding to a protein in phytochrome allows the bilitriene to "stretch" into the 5Z,10Z,15E conformation [52]. In the case of the algal C-phycocyanines the protein bound biliverdin chromophores (phycocyanobilins) are present in the "stretched" (extended) 5Z-anti-10Z-syn-15Z-anti conformations [30].



10

P; $CH_2CH_2CO_2H$

Fig. (7). Phorcabilin, a natural biliverdin with a stable extended conformation.

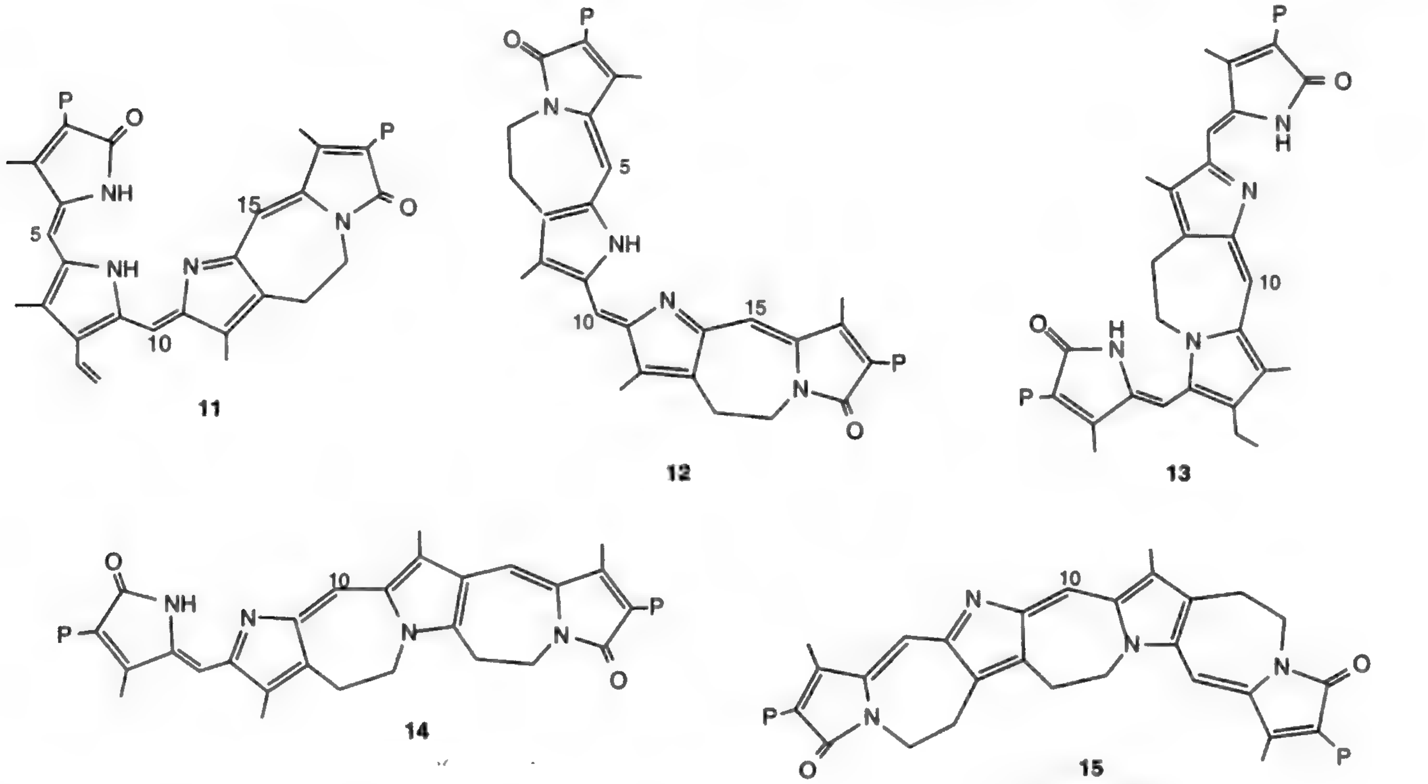
There is another structural device that can lead to stable extended biliverdins. Certain larvae and wings of adult butterflies (e.g., *Papilio sarpedon*) contain extended biliverdins due to the intramolecular addition of one or two vinyl residues to the basic pyrrole nitrogens [53]. Thus phorcabilin **10** (the pigment of the butterfly *Papilio phorcas*) has the 5Z,10E,15Z geometry in a C10 fixed conformation (Fig. **7**).

Our team has prepared by synthesis [54,55] a rather large series of extended biliverdins (**11-15**, (Fig. **8**)) which were very useful for the further understanding of the biological properties of the biliverdins (see below).

Biliverdin Reductase: Its Metabolic Regulation

Biliverdin reductase (BvR) reduces biliverdins (bilitrienes-*a,b,c*) to bilirubins (biliadienes-*a,c*) in a NADPH-dependent reaction. It has been isolated and purified from a variety of tissues where hemoproteins are degraded: rat liver and kidney [56-58], pig spleen [59], human liver [60], rat brain [61], cow spleen [62] and ox kidney [59].

Although multiple forms of the enzyme have been detected in different tissues [57,60,61] we will focus the discussion on the behavior of the molecular forms of BvR from rat liver, since its regulatory properties have been exhaustively studied. Early in the studies it was

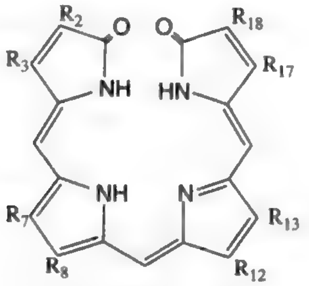


11 **12** **13** **14** **15**

P; $CH_2CH_2CO_2H$

Fig. (8). Structures of synthetic stable extended biliverdins of type IXδ.

realized that rat liver BvR was very efficient in reducing an ample spectrum of helical biliverdins. As long as the substituents included two or more propionate residues (Fig. **9**) the bilitriene was reduced by BvR to a bilirubin (biladiene-*a,c*). The rest of the possible six substituents did not influence substrate capacity [63-66].



$R_3=R_8=R_{13}=R_{18}=P$
$R_3=R_8=R_{12}=R_{18}=P$
$R_3=R_7=R_{13}=R_{18}=P$
$R_2=R_8=R_{13}=R_{18}=P$
$R_3=R_8=R_{13}=R_{17}=P$
$R_3=R_8=R_{12}=P$
$R_3=R_7=R_{18}=P$
$R_8=R_{13}=R_{17}=P$
$R_2=R_3=R_{18}=P$
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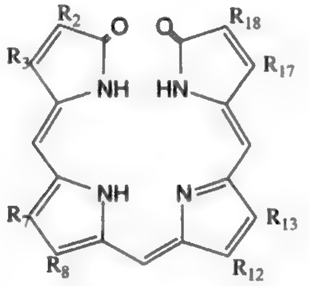
$R_2=R_{17}=P$
$R_{12}=R_{17}=P$
$R_8=R_{12}=P$
$R_{13}=R_{17}=P$
$R_2=R_{18}=P$
$R_3=R_7=P$

Fig. (9). Biliverdins that are substrates of biliverdin reductase.

Bilitrienes substituted with only one propionate residue (Fig. **10**) are not substrates of BvR, independent of the nature of the other seven possible substituents [67].



$R_7=P$
$R_8=P$
$R_2=P$
$R_{12}=P$
$R_3=P$

Fig. (10). Biliverdins that are not substrates of biliverdin reductase.

The cosubstrate NADPH could be replaced by NADH, 3-acetyl NADPH, or deamino NADPH with retention of good substrate activity for the natural type IX biliverdins [63]. BvR of livers of normal rats could be separated into two molecular forms: a major one (MF1) with a high reduction rate for biliverdin IXα **4** and a low one for biliverdin IXβ **7**, and a minor form 2 (MF2) with similar reduction rates for both isomers [68]. BvR isolated from livers of rats pretreated either with $CoCl_2$ or phenylhydrazine had a new major molecular form 3 (MF3) with a high reduction rate for the IXα and IXβ isomers, and the minor form MF2. Both $CoCl_2$ and phenylhydrazine are known to induce the chemical oxidation of hepatic hemoproteins and hemoglogin. It was shown that the appearance of MF3 was sequential in time with the disappearance of MF1 and had a half-life of 72 hr, after which resynthesis of MF1 already took place. MF3 was therefore a post-translational modification of MF1, and could be considered an epigenetic isozyme. MF1 was a monomer of MW=34kDa with three thiol residues, MF3 had 4 thiol residues and a MW=68kDa, and MF2 had MW=68kDa and six thiol residues. MF3 was transformed back into MF1 by incubation with reduced thioredoxin, while MF2 was not affected by the latter. The dimerization of MF1 to MF3 was therefore an enzymatic process where two thiol residues of MF1 are oxidized to form a disulfide bridge. This oxidation is catalyzed by a NAD^+-dependent peroxisomal dehydrogenase induced by the oxidant species released by $CoCl_2$ [69]. The dehydrogenase had MW=54kDa, did not oxidize either reduced glutathione or cystein, and did not form mixed disulfides of the latter and MF1.

The peroxisomal dehydrogenase was not induced when the rats were pretreated with phenylhydrazine, a well known hemolytic agent. The latter induced however the conversion of MF1 to MF3. It is known that phenylhydrazine is a reducing agent that in vivo is easily transformed into its diamide **16** (1,1'-azobis(N,N'-dimethylformamide)); a well known thiol oxidant that has a high reaction rate with gluthatione [70]. When the diamide **16** was given directly to rats (1.3 mg/100g), it converted MF1 into MF3 *in vivo* [71]. The conversion preceded the decrease in the content of reduced gluthatione. No NAD^+-dehydrogenase was induced. Gluthatione administered together with the diamide **16** prevented the conversion of MF1 into MF3. To prove that the latter was a chemical event even *in vivo*, MF1 was incubated with the diamide **16** and shown to be converted into MF3. The conversion was both diamide concentration-dependent and incubation time-dependent. It was thus unambiguously established that chemicals which are known to induce oxidative stress *in vivo* in rats not only induce heme oxygenase and lead to oxidative breakdown of hemoproteins [44], but also affect the isozyme pattern of liver BvR. The molecular and catalytic properties of rat liver BvR are summarized in Table I [68].

The conversion of rat liver BvR MF1 into MF3 under conditions of oxidative stress may reflect a biological situation where the chemical oxidation of hemoproteins (e.g. catalase, hemoglobin) result in the formation of

Table I. Molecular and Catalytic Properties of the Three Molecular Forms of Rat Liver Biliverdin Reductase (BvR)

Molecular Forms of BvR	2	3	1
Elution Profile of DEAE-cellulose	Early	Intermediate	Late
Molecular Weight	56,000	68,000	34,000
Substrate Specificity	IXα, β	IXα, β	IXα
Thiol Residues	6	4	3
Treatment			
In vivo:	$CoCl_2$	MF3← - - - -// - - - - -MF1	
	Phenylhydrazine	MF3← - - - -// - - - - -MF1 ↑ Inhib. by CHX or Act.D	
	Diamide **16**	MF3← - - - - - - - - - - -MF1	
	Peroxisomal Dehydrogenase + NAD^+	MF3← - - - - - - - - - - -MF1	
In vitro:	Diamide **16**	MF3← - - - - - - - - - - MF1	
	Reduced thioredoxin	MF3 - - - - - - - - - - →MF1	

higher intracellular pools of biliverdin IXβ **7**, which would require a reductase with a higher affinity for the IXβ type. It has been recently reported that a human biliverdin IXβ reductase was identified in adult livers, and could be related to the oxidative degradation of hemoglobin by activated oxygen species produced in the red blood cells [72]. The dimerization of MF1 under conditions of oxidative stress may also reflect a conformational change where the catalytic site is less exposed to oxidizing species. This possibility was explored with the help of polyclonal antibodies raised against the native purified rat liver MF1 of BvR [73]. The rationale behind this approach was based on the fact that antibodies elicited by a native protein recognize epitopes localized on its surface. If the dimerization of MF1 conceal previously exposed regions of the protein, it could be expected that this change in the protein conformation will be reflected in the masking of the antigenic determinants recognized by the anti-MF1 antibodies. Indeed, rabbit polyclonal antibodies raised against MF1 did not recognize MF3. This lack of recognition persisted even when the dimeric MF3 was denatured with SDS or urea under non-reductive conditions. Reduction of MF3 with reduced thioredoxin gave the monomeric MF1, which was fully recognized by the antibodies. Hence the antigenic domain which is exposed in MF1 became inaccessible to the antibodies upon dimerization of the enzyme to MF3.

BvR from rat spleen and kidney showed a single molecular form which was very similar to MF1 from liver, but was not converted into an MF3-like dimer [57]. By using the Western blotting techniques, it was found that the spleen and kidney enzyme cross-reacted strongly with the antisera generated against liver MF1. When equal activities of purified liver MF1 and BvR from spleen or kidney were reacted with the antiserum, the inhibition of the enzymatic activities of the reductases showed similar kinetics, suggesting that the enzyme from the three tissues share an immunospecific antigenic domain similar to that of hepatic MF1. Immunoblotting of the crude spleen enzyme revealed a polypeptide with identical mobility as that of the homogeneous kidney and liver MF1 reductases. Hence, BvR from different tissues appear to have a similar antigenic domain which becomes inaccessible upon dimerization of the liver enzyme.

The antibodies, even at high dilutions, neutralized the enzymatic activity of MF1. This inhibition was impaired by the presence of biliverdin or NADPH, suggesting that the antigenic domain of BvR may be localized near the active site or is part of the same. Modification of the lysine or arginine residues of MF1 which are involved in substrate binding (see below), impaired the interaction of the enzyme with the antibodies. Alkylation of the thiol group involved in this dimerization had no effect on the interaction of MF1 with the antibodies. It could be concluded that during the conversion of MF1 to MF3 under conditions of oxidative stress, the antigenic site of MF1 becomes buried and that the vicinal catalytic site is very likely less exposed to oxidative damage.

The Molecular Mechanism of Biliverdin Reduction by BvR

Rat liver (and kidney) BvR contains 3 thiol groups per molecule. The enzyme can be inhibited by incubation with either N-ethyl or N-phenyl maleimides (alkylation of thiols). The inhibition could be made selective by titration with increasing concentrations of N-ethyl maleimides (NEM) [74]. At low concentrations (90 mol NEM/mol BvR) the alkylating agent inhibited the thiol group involved in the conversion of MF1 into MF3. The catalytic activity of BvR remained essentially unaffected. Hence the enzyme carries an exposed thiol group involved in the metabolic regulation of the enzymatic activity but not in its catalytic activity. As the concentration of NEM increased (300 mol NEM/mol BvR), two thiol groups were alkylated and both

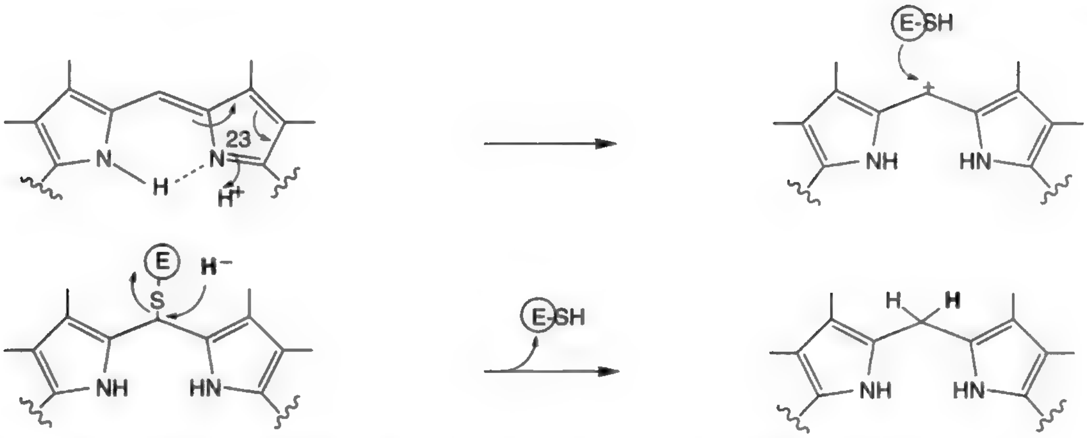


Fig. (11). Outline of the mechanism involved in the enzymatic reduction of biliverdins to bilirubins.

conversion of MF1 to MF3 and catalytic activity were inhibited. The third thiol group could not be reached with either the maleimides or iodoacetamide. Preincubation of MF1 with biliverdin and NADPH protected the thiol group involved in the catalytic activity from alkylation and prevented its inhibition (but not its conversion to MF3). It was obvious that biliverdin binds to a thiol group and that NADPH also binds in its close vicinity. Even addition of guanidine hydrochloride could not cleave biliverdin from its binding site on the enzyme. That this binding takes place through a thiol residue was shown by spectroscopic evidence. It was known from biliverdin chemistry [75] that nucleophiles (e.g., thiols) add to the C-1O methine bridge to form a thiobilirubinoid adduct (Fig. **11**). On addition of 2-mercaptoethanol or cysteine the blue color of the biliverdin solution changes to the yellow color of the bilirubinoid adduct (λ max:420 nm). On addition of biliverdin to BvR (in 20 mM phosphate buffer) the formation of the bilirubinoid adduct on the enzyme could be detected by its λ max at 421 nm. [74].

Spectroscopic data also helped to secure the binding domain of NADPH to BvR. It was known from extensive studies with NADH-dependent dehydrogenases (alcohol dehydrogenase, lactate dehydrogenase) that when the nucleotide binds to the enzyme its UV max shifts from 340 mm to 325 mm. This hypsochromic shift is due to the presence of a positive charge at a distance of ca. 3Å of the dihydropyridine ring [76]. Similar shifts were obtained when NADPH and 3-acetyl-NADPH were used as BvR cosubstrates [74]. The positive charge was identified as an ε-lysine amino group which was located within the 3Å range of the thiol residue involved in the catalytic activity. Both the cysteine residue and the lysine residue reacted with o-phtalaldehyde to give a fluorescent isoindole derivative bound to BvR. The increase in isoindole formation paralleled the decrease in BvR activity [74]. More evidence regarding the essential amino acids involved in the catalytic activity were obtained from preincubation studies with butanedione and phenylglyoxal. They revealed that arginine residues are involved in NADPH binding and are necessary for the enzymatic activity. When putting together the evidence obtained from the chemical modifications and from the spectroscopic data, a tertiary model of the transition state of BvR (MF1) could be outlined (Fig. **12**).

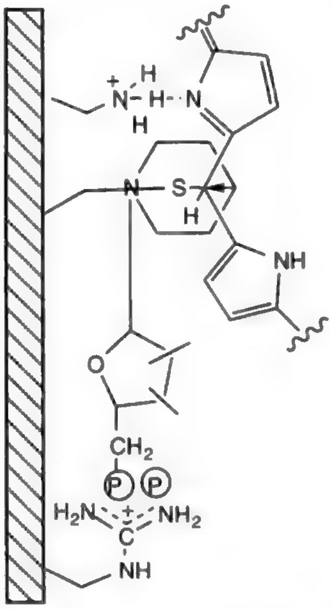
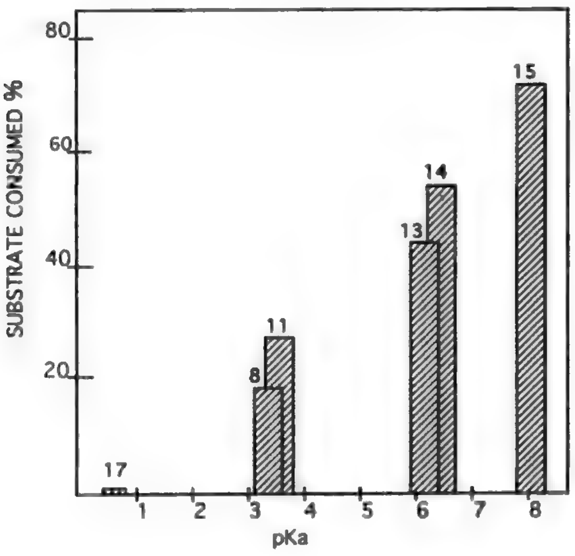


Fig. (12). Tertiary model of the transition state of biliverdin reductase.

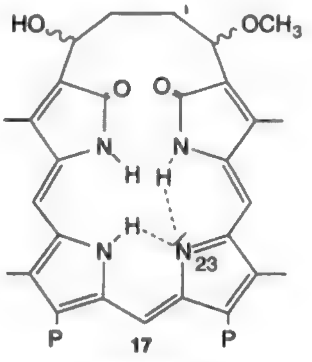
A thiol residue adds to the sp^2 C-10 meso bridge of biliverdin to give a sp^3 tetragonal intermediate. The "pull-push" mechanism is very likely provided by the neighboring positively charged residue which enhances the electrophilic character of C-10. The cosubstrate is held close to the S-bilirubinoid adduct by the electrostatic interaction of the pyrophosphates and the arginine residues. The 4-hydride is transferred to the C-10 tetragonal carbon, the sulfur bond is cleaved, and a bilirubin derivative is obtained [74]. The recently reported amino acid sequence of the enzyme [58] revealed that indeed the cysteine residues (cys-280 and cys-291) are flanked by sequences of positively charged amino acids, rich in lysine and arginine residues. This could also explain the need of two negatively charged propionate residues for a bilitriene to be a substrate of BvR (see above). They very likely interact with the cluster of positively charged amino acids surrounding the cysteine residues to fix the substrate at the active site domain.

A new light was shed on the biological mechanism of biliverdin reduction when the extended biliverdins **11-15** (Fig. **8**) were assayed as substrates of BvR. It was found that the fully extended biliverdin **15** was reduced at the highest rate followed by the partially extended biliverdins **14**, **13**, **12**, **11** which were reduced at decreasing rates, while the helical biliverdin **8** (Fig. **3**) was reduced at the slowest rate [77]. This order of enzymatic rates was paralleled by the rates of formation of their thio bilirubinoid adducts with 2-mercaptoethanol, as well as with the rates of chemical reduction with sodium borohydride (hydride release) [77]. It was evident that in the extended biliverdins the rates of nucleophilic additions to C-10 are higher than in the helical conformers. To understand the structural basis for this enhanced electrophilicity at C-10, it suffices to keep in mind the outline of the mechanism proposed for the enzymatic reduction of biliverdins to bilirubins (Fig. **11**). Protonation at N23 leads to a concurrent formation of a mesomeric species positively charged at C10. This species is then attacked by the nucleophiles, first by the thiol residue and then by hydride anion released from the NADPH. As the basicity of N23 increases the reduction rates of the biliverdins should also increase. The pKa's of the helical and extended biliverdins were therefore measured and correlated with their reduction rates (Fig. **13**) [78].



(Fig. 13). Enzymatic reduction rates of biliverdins **8**, **11**, **13**, **14**, **15**, and **17** as a function of their pKa's.

It was evident that "stretching" increased the basicity of the N23 pyrrolenine nitrogen, which was reflected in an enhanced substrate capacity toward BvR. The formation rates of the thiobilirubinoid adducts with 2-mercaptoethanol and the reduction rates with sodium borohydride also increased with pKa increase. A biliverdin such as **17** (Fig. **14**), which is forced into an obligate helical conformation, had a pKa = 0.4 and was not a substrate of BvR (Fig. **13**).



P; $CH_2CH_2CO_2H$

Fig. (14). Biliverdin with an obligate helical conformation.

The increase in the pKa values reflects an increase in the localization of the pyrrolenine nitrogen (N23) lone pair. An alkyl substituted dipyrrylmethene (a dimer) is a relatively strong base (pKa = 8.5) due to the basicity of its pyrrolenine nitrogen. Upon increasing the delocalization of the nitrogen lone pair of electrons in the pyrrolenine by extension of the conjugated system, the basicity of the pyrrolenine ring decreases (to pKa 6.0 in tripyrrinones (trimers), pKa = 4.0 in helical biliverdins (tetramers), pKa ca. 2.5 in pentapyrrins (pentamers)). In an extended biliverdin such as **15** the conjugated system present in **4** is almost completely interrupted, and therefore the pyrrolenine basicity is again of the same order of magnitude as in a dipyrrylmethene.

The increased basicity of the bilitriene is the driving force of the enzymatic reduction by BvR. However, stereochemical factors are also of importance. It is known that the dehydrogenases are among the more stereospecific enzymes in their interaction with substrates [79]; and BvR resembles a nucleotide-dependent dehydrogenase in its mechanisms of action (Fig. **12**). We already mentioned that a helical biliverdin is a 1:1 mixture of P/M enantiomers of opposite helical sense. We also mentioned that when a protein binds non-covalently to a biliverdin it binds to only one of the enantiomers, which retains its all-Z helical configuration but could adopt a more extended anti, syn, anti conformation [80]. We therefore proposed [77] that: a) BvR binds only one of the P/M enantiomers; b) that on protonation of N23 by the enzyme the biliverdin adopts an extended conformation; and c) that the extended conformation favors its reduction to bilirubin. A rapid interconversion of the P/M enantiomers in solution follows, until all biliverdin is reduced to bilirubin. It has been recently shown [81] that albumin-bound biliverdin IXα **4**, a complex which has preference for the (P)-shaped conformational enantiomer, is electrochemically reduced to the corresponding albumin-bound bilirubin IXα **5** with an M-chiral conformation. Similar results were obtained with sodium borohydride reduction of albumin-bound biliverdin IXα **4** [86]; thus lending support to our suggestions that BvR very likely confers enantioselectivity to the enzymatic reduction of biliverdins to bilirubins.

Concluding Remarks

Biliverdins are linear oligopyrroles with either helical or extended conformations. In bile pigments they preferentially have the helical conformation and are a 1:1 mixture of right-handed and left-handed "locker-washer" type enantimoers. They are reduced to bilirubins by a soluble biliverdin reductase which was found to be modulated in rat liver depending on the physiological conditions which regulate heme and hemoprotein breakdown. Under conditions of normal heme catabolism, biliverdin IXα **4** is the main oxidation product of heme; under conditions of oxidative stress hemoproteins are degraded to other biliverdin IX isomers. Abnormal hemoglobins are also oxidized to biliverdin isomers other than the usual IXα isomer. Rat liver biliverdin reductase was found to exist in three different molecular forms with different production rates toward the biliverdin isomers. The molecular forms interconvert according to the prevalent physiological conditions. This interconversion secures that the different biliverdin isomers will be efficiently reduced to bilirubins. The regulatory properties of the enzyme together with its broad substrate specificity are directed to achieve the complete reduction of biliverdins to bilirubins under the most different physiological conditions. Biliverdin reductase is therefore an adaptable enzyme which will convert to bilirubins a large number of either natural or synthetic biliverdins and thus secure their prompt elimination and excretion from the organism in mammals.

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